1970

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RESISTANCE TO BORDETELLA BRONCHISEPTICA INFECTION IN SWINE

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Signature was redacted for privacy.

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Iowa State University
Ames, Iowa

1970

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INTRODUCTION

In 1956, it was reported that pure cultures of Bordetella bronchiseptica, when instilled into the nasal cavities of young experimental swine, would cause turbinate hypoplasia and pneumonia. Research in swine respiratory diseases since 1956 has shown B. bronchiseptica to be the main etiologic factor in the disease commonly called atrophic rhinitis.

During the past decade, Bordetella rhinitis has been well characterized. Studies on the pathogenesis of Bordetella rhinitis have shown that experimental infection of swine, 4 weeks of age or less, readily produces turbinate hypoplasia by 2-5 weeks postinoculation. Microscopic changes include hyperplasia and metaplasia of the pseudostratified ciliated columnar epithelium, cilia swelling and loss, infiltration of neutrophils and mononuclear leucocytes into the lamina propria, submucosal fibroplasia, and osseous resorption and replacement fibrosis. The B. bronchiseptica organisms remain upon the epithelial cell surface and do not invade the submucosa.

Pathogenicity for pigs of various isolates of B. bronchiseptica has been compared. Intranasal inoculation of 4 isolates of swine origin, an isolate of rabbit origin, an isolate of cat origin, and an isolate of rat origin caused mild to moderate turbinate hypoplasia in experimental swine. An isolate of dog origin caused no turbinate hypoplasia. This series of experiments helps explain the variation of the degree

of turbinate hypoplasia reported in some field outbreaks of rhinitis. Also,the possibility of other animal species serving as reservoirs of infection was illustrated.

The role of Pasteurella multocida in rhinitis of swine has recently been more clearly elucidated. This organism occurs at a low incidence in the nasal cavities of North American swine. Canadian workers have demonstrated that P. multocida, serotype B, would cause rhinitis and turbinate hypoplasia by intranasal inoculation of swine with the organism. However, P. multocida, serotype D, is the most commonly occurring strain in the United States. Experimental infection of swine with serotype D was only accomplished by preconditioning the nasal cavity with B. bronchiseptica infection for 1 week. The dual experimental infection of the swine nasal cavity by both bacteria enhances the degree of turbinate hypoplasia as compared to the swine nasal cavity infected with B. bronchiseptica alone.

In the early 1960's, bacteriologic examination of the nasal cavities of swine from herds affected with clinical signs of rhinitis revealed that B. bronchiseptica existed in 56 to 68 percent of the herds surveyed. Progressive purebred and commercial herds had an incidence ranging from 38 to 54 percent. Shortly after these surveys were conducted, it was discovered that certain sulfonamides (sulfamethazine and sulfaethoxypyridazine) when added to the swine ration would clear B. bronchiseptica infection from the swine nasal cavity in 3-5 weeks.

These sulfonamides have been incorporated with growth promoting antibiotics and have enjoyed wide popularity as feed additives to swine rations for the past several years.

In 1967, the nasal cavities of swine from 102 progressive Iowa swine herds were cultured bacteriologically. Results of this survey indicated that 25 percent of the herds had B. bronchiseptica and 9 percent had P. multocida. The isolates of B. bronchiseptica recovered from 20 herds (80 percent) were resistant to sulfonamide as determined by sensitivity disc assay. These results indicate that the decrease in the number of herds infected with B. bronchiseptica since 1962 may be attributed to the widespread use of sulfonamide as a feed additive. However, the incidence would probably be lower had not sulfonamide resistant strains of the organism appeared. According to the 1967 survey, approximately 20 percent of the Iowa swine herds are infected with B. bronchiseptica which are resistant to sulfonamides. The presence of drug resistant strains of B. bronchiseptica indicates the need for either additional drugs with the ability to clear the organism from the nasal cavity or immunizing products for prevention of infection.

Recently it was demonstrated that a nasal resistance to reinfection with B. bronchiseptica could be induced in swine by either clearing the nasal cavity of a virulent strain infection by drug therapy or previous infection of the nasal cavity

with a low-virulence strain of the organism. After clearing the low-virulence strain (4-6 weeks after initial exposure) experimental swine were resistant to subsequent challenge to virulent strain for at least 95 days.

In view of the need for methods of controlling Bordetella rhinitis, the present study was undertaken to investigate the mechanism by which respiratory resistance to B. bronchiseptica infection was induced in experimental swine and to evaluate possible application of these findings to the control of this disease.

REVIEW OF THE LITERATURE

This literature review covers four areas: 1. Cultural Characteristics of Bordetella bronchiseptica, 2. Bordetella bronchiseptica Infection in Swine, 3. Resistance of Various Animals to B. bronchiseptica and Bordetella pertussis, and 4. Respiratory Tract Resistance to Infectious Agents. Excellent reviews by Winsser (1960) and Ross (1965) cover the history, bacteriology, and animal pathogenicity of B. bronchiseptica; therefore, in this review only the more pertinent literature in these areas will be discussed.

Cultural Characteristics of Bordetella bronchiseptica

Bordetella bronchiseptica was placed in the genus Bordetella in both the seventh edition of Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) and the fifth edition of Topley and Wilson's Principles of Bacteriology and Immunity (Wilson and Miles, 1964). Ferry (1910) was the first to isolate the organism. He names it Bacillus bronchicanis since it was isolated from the respiratory tract of dogs with distemper. After being found in the respiratory tract of a variety of animals, Ferry (1912a) renamed the organism Bacillus bronchisepticus.

Prior to 1957, B. bronchiseptica had been placed in three different genera: Alcaligenes (Bergey, 1923), Brucella (Bergey et al., 1939; Breed et al., 1948), and Haemophilus

(Wilson and Miles, 1946, 1955). Inclusion in the genus Alcaligenes was based on morphologic, growth and biochemical similarities to Alcaligenes faecalis. Placement in the genera Brucella and Haemophilus was based on morphologic, growth, biochemical and antigenic similarities to members of those genera.

Ferry and Noble (1918) and Ferry and Klix (1918) reported the antigenic relationship of B. pertussis and B. bronchiseptica. Further studies have indicated close antigenic, morphologic, and biochemical similarities between B. parapertussis, B. pertussis, and B. bronchiseptica (Eldering and Kendrick, 1938; Bruckner and Evans, 1939; Eldering, 1941; Keogh et al., 1947). These three species were classified in a new genus Bordetella by Moreno-Lopez (1952). Justification for this classification was further documented by Andersen (1952); Lacey (1953; Eldering et al. (1957); Sutherland and Wilkinson (1963); and Barber et al. (1967).

Bordetella bronchiseptica grows as a pleomorphic slender rod in broth and as a coccoid rod on agar. The bacillary forms measure approximately 0.5 by 1.5 micron and the coccoid forms measure approximately 0.5 by 1 micron (McGowan, 1911; Torrey and Rahe, 1913; and Lautrop and Lacey, 1960). Ferry (1910, 1911) reported that B. bronchiseptica was Gram negative and was bipolar when stained with Loeffler methylene blue. Evans and Maitland (1939) and Repentigny and Frappier (1956) demonstrated capsules on B. bronchiseptica.

Richter and Kress (1967) studied the fine structure of B. bronchiseptica and found it to have a cell wall composed of five layers, a trilaminar cell membrane, many ribosomes in the cell matrix, and nucleoidal material composed of fibrils and dense bodies. Ferry (1910), Smith (1913), and Torrey and Rahe (1913) found B. bronchiseptica to be motile by means of peritrichous flagella. Lautrop and Lacey (1960) and Ross (1965) reported that some strains of B. bronchiseptica are nonmotile. Using electron microscopy, Labaw and Mosley (1955) interpreted metal-coated preparations of flagella to exist in a counter-clockwise or left-handed triple helix. By comparison, recent observations by Richter and Kress (1967), using both metal-coating and negative staining techniques, have shown images of B. bronchiseptica flagella to exist as a "braided" structure. Negative contrast preparations indicated that 5 to 6 strands were interlaced to form each "braid" with a strand width of 20 Å. The flagella width ranged from 180 to 220 Å.

Torrey and Rahe (1913) reported that B. bronchiseptica alkalized carbohydrates and litmus milk but did not ferment carbohydrates; some strains reduced nitrates to nitrites, and the organism did not produce indole or hydrogen sulfide. Ross (1965) found that 17 of 60 strains reduced nitrates to nitrites. Szturm and Bourdon (1948) showed that B. bronchiseptica utilizes citrate as the sole source of carbon. Bradford and Slavin (1937) reported the organism produced catalase. Lautrop and Lacey (1960) and Farkas-Himsley (1963) found that B. bronchi-

septica produced cytochrome oxidase, and the latter author observed it was lysine decarboxylase positive. Ulrich and Needham (1953) found that the organism required nicotinic acid for growth in a vitamin free synthetic medium and that B. bronchiseptica produced urease. This latter characteristic aids in the differentiation of B. bronchiseptica from Alcaligenes faecalis.

Sutherland and Wilkinson (1963) reported that B. bronchiseptica produces a "blue protein" called azurin which undergoes reduction in the presence of concentrated cell-free extracts of the organism and succinate. These workers postulated that azurin may act as an alternative in the electron-transport system between cytochrome c and cytochrome oxidase.

Bordetella bronchiseptica has been reported to be beta hemolytic on the following types of blood agars: bovine (Ross et al., 1963a), horse (L'Ecuyer et al., 1961), sheep (Winsser, 1960), and rabbit, dog, and guinea pig (Torrey and Rahe, 1913). The organism does not produce hemolysis of washed rabbit, sheep, or human erythrocytes when incorporated in broth culture medium (Winsser, 1960). L'Ecuyer et al. (1961) reported that transfer of B. bronchiseptica in embryonated hens eggs increased the degree of hemolysis on horse blood agar.

Three types of agglutinogens of B. bronchiseptica have been described: flagellar H antigens, heat labile K antigens, and heat stable 0 antigens (Eldering et al., 1957 and Lautrop

and Lacey, 1960). Fourteen heat labile K antigens are shared by B. bronchiseptica, B. pertussis, and B. parapertussis, but each species contains at least one specific antigen. Differentiation of species by heat stable O antigens has not been reported. Keogh et al. (1947) reported that whole B. bronchiseptica cells, filtrates, and supernatants of broth cultures would cause agglutination of human, mouse, and fowl erythrocytes. This hemagglutinin was also present in B. pertussis and B. parapertussis cells and cultures. Bordetella bronchiseptica was found to agglutinate sheep erythrocytes but not horse erythrocytes (Joubert et al., 1960). Gallagher (1965) reported that an isolate of B. bronchiseptica from a horse agglutinated horse and parakeet erythrocytes.

Eldering (1941) prepared lipopolysaccharides from the three Bordetella species by extraction of whole cells with trichloroacetic acid. The lipopolysaccharides were toxic for mice and rabbits. Harris et al. (1968) reported that lipopolysaccharides extracted with trichloroacetic acid from B. bronchiseptica and B. pertussis were toxic for embryonated hens eggs and produced an inhibition of some of the energized processes of beef heart mitochondria in vitro. This lipopolysaccharide specifically acted by inhibiting oxidative phosphorylation at site 3 in complex IV but prevented electron transport just prior to complex IV in mitochondria. It is interesting to note that the azurin described by Sutherland and Wilkinson (1963)

may have the opposite activity of lipopolysaccharide in the electron transport system. Phenol extraction procedures were utilized to isolate lipopolysaccharides from the three species of Bordetella (Maclennan, 1960). He found that Bordetella lipopolysaccharides were similar both chemically and biologically to those of other Gram negative bacteria. Evans and Maitland (1939) demonstrated a heat labile toxin in freeze-thaw extracts of B. bronchiseptica and B. pertussis. These preparations were toxic to guinea pigs and caused dermonecrosis when injected subcutaneously into rabbits. The toxin was formalin sensitive, nonantigenic, and lost potency on filtration through a Seitz E K filter. Oddy and Evans (1940) reported that the heat labile toxin induced initial hyperglycemia and later hypoglycemia and death in rabbits. The hypoglycemia inducing factor was eliminated by heating the toxin at 55° C for 30 minutes.

Rauch and Pickett (1961) reported that 38 bacteriophages were obtained from 48 strains of B. bronchiseptica as spontaneous mutants without ultraviolet induction. Ultraviolet exposure of lysogenic organisms failed to cause induction. Thirty-five of the 48 strains of B. bronchiseptica were sensitive to at least one phage. Bordetella parapertussis was also sensitive to these phages; however, B. pertussis was not. Litkenhous and Liu (1967) isolated 2 bacteriocins from 24 strains of B. pertussis which inhibited the growth of 20 strains of the same organism. These 2 bacteriocins were inactive on

B. bronchiseptica and B. parapertussis.

Bordetella bronchiseptica has been commonly associated with diseases of the respiratory system in animals and on rare occasions, poultry. The organism has been isolated from the following: dogs (Ferry, 1910); humans, monkeys, goats, cats, rabbits, ferrets, and guinea pigs (McGowan, 1911); mice (Keegan, 1920); swine (Dorset et al., 1922); fox (Rosenow, 1931); rats (Bordon and Kulp, 1939); hedgehog (Edwards, 1957); horses (Gallagher, 1965); skunk, oppossum, and raccoon (Switzer et al., 1966); and turkeys (Filion et al., 1967). Brown (1926) reported isolation of B. bronchiseptica from a child with clinical symptoms of whooping cough. A rabbit was considered to be the source of infection for the child. Although B. pertussis has been the most consistent organism isolated from whooping cough in man, B. bronchiseptica has been found associated with the disease occasionally. Estimates indicate that 0.1 percent of the whooping cough cases in London were caused by B. bronchiseptica (Lautrop and Lacey, 1960).

Bordetella bronchiseptica Infection in Swine

The role of B. bronchiseptica in the respiratory disease complex of swine has only recently been elucidated and substantiated. Early reports mention B. bronchiseptica or organisms with similar characteristics associated with pneumonia in swine (Dorset et al., 1922; Spray, 1922; and Thorp and Tanner, 1940).

Phillips (1943) considered B. bronchiseptica to be a significant primary agent in swine pneumonia; the organism was isolated from swine on 7 farms in Ontario. Phillips (1944) reported increased protection against hemorrhagic septicemia if B. bronchiseptica and Pasteurella multocida antisera were administered to baby pigs at birth. A bacterin containing both organisms was used later. Betts (1952) recovered B. bronchiseptica from pneumonic swine lungs in England and considered the organism to be a secondary invader of virus pneumonia of pigs. Ray (1950) found B. bronchiseptica to be associated with pneumonia in swine in the United States and believed a bacterin containing the organism protected young pigs. Ray (1959) suggested the term porcine whooping cough for swine pneumonia caused by B. bronchiseptica and believed infected dogs served as a reservoir of infection for swine.

Evidence that B. bronchiseptica is a primary cause of pneumonia in swine has been reported from France (Joubert et al., 1960), United States (L'Ecuyer et al., 1961, and Dunne et al., 1961), England (Goodwin and Whittlestone, 1962 and 1964), and Norway (Årskog, 1967). L'Ecuyer et al. (1961) reproduced pneumonia with B. bronchiseptica by a combination of intratracheal and intranasal inoculations of the organism grown in embryonating chicken eggs. These workers described the macroscopic and microscopic changes occurring in this pneumonia at 11 days postinoculation which included: a bluish tan coloration to the pneumonic areas, hyperemia of the peribronchial

vessels, lymphocytic infiltration of the propriomucosa, and alveolar exudate consisting of neutrophils, lymphocytes, edema, and fibrin. Duncan et al. (1966a) described the lesions produced in experimental pigs following intratracheal inoculation with broth cultures of the organism. The pigs were necropsied at 2, 4, 6, 8, 12 days and 4 weeks postinoculation. Macroscopically, the color of the pneumonic areas changed from a dark red to brown to yellow to a greyish yellow as the interval postinoculation increased. The pneumonic areas became firm to palpation as the lesion became more chronic. Microscopically, early lesions were hyperemic with some alveolar hemorrhage. Fibrin was present in the interalveolar spaces which were swollen by edema. Neutrophils were present in the interlobular lymphatics and the lumens of the bronchi and bronchioles. Bacterial colonies were present on the mucosal surface. Metaplasia of the bronchiolar epithelium occurred by 6 days postinoculation. As the postinoculation interval increased, epithelialization of the alveoli occurred, adventitial fibroplasia of the bronchioles occurred, cellular exudate increased and the degree of hemorrhage and hyperemia decreased.

Daugherty (1941) recovered B. bronchiseptica from the nasal cavities of pigs afflicted with rhinitis and observed that a clinical improvement could be obtained from administrations of a mixed canine bacterin containing the organism. Phillips (1946) and Moynihan (1947) recovered B. bronchiseptica from the nasal cavities of swine with atrophic rhinitis.

Switzer (1956) presented evidence that various etiologic agents were involved in the production of atrophic rhinitis in swine. He found that a filter-passing agent (Selas No. 01 and 0.5-0.75μ cellulose membrane), Pasteurella multocida, or Alcaligenes sp. would produce turbinate atrophy when instilled intranasally into young pigs. Claflin (1958) reported that Alcaligenes faecalis would produce atrophic rhinitis. Later reports by Switzer (1959) and Cross and Claflin (1962) indicated that the organism originally identified as Alcaligenes sp. was B. bronchiseptica. These workers produced atrophic rhinitis in pigs by instilling broth cultures of B. bronchiseptica into the nasal cavity. Cross and Claflin (1962) isolated the organism from pigs with atrophic rhinitis within 10 herds. Dunne recovered B. bronchiseptica from the nasal cavity of a pig suffering from pneumonia caused by the same agent.

Ross (1965) and Duncan (1965) reported on an extensive study of the microbiology, serology, pathology, and pathogenesis of atrophic rhinitis caused by experimental inoculation of swine with B. bronchiseptica. Using respiratory disease free swine as their experimental animal, 1 group of swine were inoculated at 3 days of age and a second group of swine were inoculated at 4 weeks of age with B. bronchiseptica. The virulence of the organism had been enhanced by passage in embryonating chicken eggs. The first group was necropsied in subgroups at 1, 2, 3, 5, and 20 weeks postinoculation. The

second group was necropsied in subgroups at 1, 2, 3, 4, 6, and 8 weeks postinoculation. In group 1, 94 percent of the animals necropsied between 2 and 5 weeks postinoculation had turbinate atrophy and in group 2, 66 percent of the animals necropsied between 2 and 8 weeks postinoculation had turbinate atrophy (Duncan et al., 1966b and Ross et al., 1963a). The organism was consistently recovered from the nasal cavity at necropsy in both groups through 5 and 8 weeks postinoculation respectively. Of the 11 pigs which were necropsied from group 1 at 20 weeks postinoculation, only 2 pigs were positive for B. bronchiseptica in the nasal cavity (Ross, 1965). Seven of these 11 pigs exhibited macroscopic lesions of distorted turbinates due to atrophy and regeneration (Ross, 1965 and Duncan, 1966b).

Ross (1965) also conducted plate and tube agglutination tests from the sera collected at necropsy of both groups. Detectable serum agglutinin, specific for B. bronchiseptica, did not occur in group 1 until 20 weeks postinoculation and in group 2 until 6 and 8 weeks postinoculation. Duncan (1965) and Duncan et al. (1966b) described the microscopic lesions of the nasal turbinate occurring in these 2 groups of experimentally infected swine. The primary lesions were hyperplasia and metaplasia of the epithelium, fibrosis of the lamina propria, neutrophil and mononuclear infiltration of the lamina propria, and osseous resorption and replacement fibrosis. The B. bronchiseptica organism was demonstrated on the epithelium among

the cilia of the turbinates and trachea by fluorescent antibody staining technique but the organism did not appear to invade the underlying tissue. Duncan and Ramsey (1965) examined the swine turbinate epithelium by electron microscopic techniques and discovered that the B. bronchiseptica infection caused the cilia to be polyhedral in shape due to swelling and the cilia to be fewer in number and spaced further apart. Based on these pathogenesis studies Duncan et al. (1966b) made the following statement:

> "The organism must produce or be responsible for the elaboration of some substance which diffuses into the tissue and elicits the change in the osseous core without causing a marked inflammatory reaction."

These workers postulated that endotoxins could be responsible for the lesions described.

Variation in pathogenicity of different strains of B. bronchiseptica for pigs has been reported (Ross et al., 1967). Intranasal inoculation of pigs 3 days of age with 4 swine isolates, a rabbit isolate, a cat isolate, and a rat isolate caused mild to moderate turbinate atrophy. An isolate of dog origin (D-1) caused no turbinate hypoplasia. The organism was isolated from the nasal cavities of all experimental swine at necropsy but in lower numbers in the pigs inoculated with the rat, dog, and rabbit isolates. Serum agglutinin was detected in samples collected at necropsy from the pigs inoculated with the swine (3 of the 4 strains), rabbit, and rat isolates.

The role of P. multocida in rhinitis of swine has recently been more clearly elucidated (Harris and Switzer, 1968). Pasteurella multocida, serotype B, has been shown to cause rhinitis and turbinate atrophy in Canadian swine (Gwatkin et al., 1953). However, P. multocida, serotype D, was reported to be the most common serotype to occur in swine (Carter, 1957). Harris and Switzer (1968) reported that experimental infection of swine with serotype D was only accomplished after preconditioning the nasal cavity with B. bronchiseptica infection for one week. The dual experimental infection of the swine nasal cavity by both bacteria enhanced the degree of turbinate atrophy as compared to swine affected with B. bronchiseptica alone.

Since the early 1960's B. bronchiseptica has often been isolated from the swine nasal cavity. Cross and Claflin (1962) isolated the organism from swine in 10 Indiana herds which contained pigs affected with atrophic rhinitis. Ross (1963 and 1965) reported that, based on pigs from 87 herds submitted to the Iowa Veterinary Diagnostic Laboratory for various reasons, 38 percent of the herds were positive for B. bronchiseptica and that of the 34 pigs positive for the organism, 29 percent had turbinate atrophy. In another survey of nasal mucus samples submitted from pigs suspected of having rhinitis, 56 percent of 32 herds were infected with B. bronchiseptica. In a second survey of nasal mucus collected from 28 Iowa swine herds suspected of having rhinitis, 68 percent of the herds were

infected with the organism (Ross, 1963 and 1965). Ross et al., (1963b), and Ross (1965) collected nasal exudate from four 8- to 10-week-old purebred swine from each of 87 herds in Iowa. Fifty-four percent of these herds were infected with B. bronchiseptica.

Shortly after these surveys were conducted, it was discovered that addition of sulfonamides (sulfamethazine and sulfaethoxypyridazine) to the swine ration at the level of 100 grams per ton would clear B. bronchiseptica from the nasal cavity of swine 3-5 weeks (Switzer, 1963). These sulfonamides have been incorporated with growth promoting antibiotics and have enjoyed wide popularity as feed additives for swine rations (Harris et al., 1967). In 1967, the nasal cavities of four 6- to 8-week-old purebred swine from each of 102 herds in Iowa were cultured bacteriologically (Harris et al., 1969). Results of this survey indicated that 25 percent of the herds had B. bronchiseptica and the isolates of the organism recovered from 20 herds (80 percent) were resistant to sulfonamides as determined by sensitivity disc assay. Sulfonamide resistant strains of B. bronchiseptica were isolated repeatedly from swine in a herd undergoing therapy with sulfonamides at recommended levels (Harris and Switzer, 1968). Harris et al. (1969) concluded that the high incidence of sulfonamide resistant B. bronchiseptica in Iowa swine:

> "...presents an imposing barrier to the control of atrophic rhinitis by therapy and emphasizes the need for other methods of control."

Complete nasal resistance to B. bronchiseptica may be established in swine by either the previous infection by a virulent strain (B) and clearance by sulfonamide therapy or the previous intranasal exposure to a low-virulence strain (D-1) (Harris and Switzer, 1969). After clearing the low-virulence strain infection (4-6 weeks after initial exposure) experimental swine are resistant to subsequent challenge to virulent strain for at least 95 days. Intranasal challenge with virulent B. bronchiseptica, given 16 times during a 53-day period, did not cause respiratory infection in resistant swine.

Resistance of Various Animals to Bordetella bronchiseptica and Bordetella pertussis

Ferry (1911) reported the first immunization studies utilizing B. bronchiseptica, the organism then believed to be the cause of canine distemper. Nine dogs were given a live broth culture and 17 dogs were given a killed suspension (1 percent formaldehyde) of B. bronchiseptica subcutaneously. These dogs were challenged by natural exposure to canine distemper at the same time as 8 unvaccinated control dogs. All of the controls died and all immunized dogs remained clinically normal. Serum agglutinins were detected in both infected and vaccinated dogs. McGowan (1911) obtained similar protection to natural exposure by means of subcutaneous injection of live organisms. Formaldehyde inactivated B. bronchiseptica bacterins were

reported to protect rabbits and guinea pigs against an epizootic of respiratory disease assumed to be caused by the organism (Ferry, 1914). Ferry and Hoskins (1920) prevented snuffles in rabbits with a bacterin containing B. bronchiseptica, Bacterium lepisepticum, and Staphylococcus albus. Wickert et al. (1958) prepared bacterins containing heat killed B. bronchiseptica (60° C for 20 minutes) preserved with 0.05 percent formaldehyde. The organism had been isolated from pneumonic lesions in rats. In intranasally challenged rats, 1 of 50 vaccinated rats (injected subcutaneously) died while 18 of 57 unvaccinated rats died. In natural exposure experiments, 2 of 247 vaccinated rats died while 9 of 247 unvaccinated rats died. Serum agglutinins were detected in both infected and vaccinated rats. Phillips (1944) and Ray (1950) reported that a bacterin containing B. bronchiseptica prevented pneumonia associated with the organism in swine. Daugherty (1941) reported that swine with rhinitis showed a clinical improvement when treated with a mixed canine bacterin containing B. bronchiseptica. In all of these studies there was no attempt to recover B. bronchiseptica from the respiratory secretions of vaccinated animals.

One of the first attempts to study the mechanism of respiratory immunity was conducted by Bailey who utilized the nasal infection of rabbits (Bailey, 1927). Sera from rabbits infected intranasally with B. bronchiseptica contained antibody detectable by agglutination but not by complement fixation.

Rabbits infected intranasally with a heavy concentration of the organism had higher serum agglutinin titers. Phagocytosis of B. bronchiseptica by segmented neutrophils occurred in the presence of sera from both carrier and non-carrier rabbits. The author arrived at the following conclusion:

> "In considering the mechanism of respiratory immunity, our results lead us to conclude that both local and general resistance play important roles in determining whether definite infection will result."

Bull and McKee (1928) reported that pneumococci transiently infect the rabbit nasal cavity while B. bronchiseptica occurs as a more chronic infection. The pneumococcal carrier state ended with the appearance of complement fixing antibody in the serum. Rabbits injected subcutaneously with both live and heat killed suspensions of pneumococci resisted intranasal exposure to the organism. The serum of the vaccinated rabbits contained complement fixing antibody. By contrast, rabbits injected subcutaneously with heat killed suspensions of B. bronchiseptica produced high titers of complement fixing antibody but did not resist intranasal challenge by the same organism.

Ganaway et al. (1965) prevented guinea pig pneumonia, caused by B. bronchiseptica, by the use of a bacterin prepared from the organism inactivated with 0.15 percent formaldehyde. Two-hundred and eighty-eight guinea pigs were injected once intramuscularly with the bacterin mixed with equal parts Freund incomplete adjuvant. The vaccinated animals were exposed

naturally to B. bronchiseptica and no deaths occurred in them. The respiratory secretions from 10 guinea pigs necropsied 7 months after vaccination did not contain B. bronchiseptica. The unvaccinated group containing 293 guinea pigs had many deaths due to pneumonia,and B. bronchiseptica was recovered frequently from the respiratory secretions. Serum agglutinins were demonstrated from the vaccinated guinea pigs.

Several workers have demonstrated the relatedness of B. bronchiseptica and B. pertussis by cross immunization. Evans and Maitland (1939) injected guinea pigs subcutaneously with a heat killed suspension (56° C for 30 minutes) of B. pertussis and challenged them intranasally with a culture of B. bronchiseptica. Three of 6 unvaccinated control guinea pigs died and none of 7 vaccinated guinea pigs died. Lung cultures from all control guinea pigs were positive for B. bronchiseptica, while the lungs of the vaccinated guinea pigs were negative. Antibody was detected in sera from the vaccinated guinea pigs by both agglutination and complement fixation tests. Eldering (1941, 1942) utilized the mouse to study cross protection of Bordetella species. She (Eldering, 1941) injected mice subcutaneously with merthiolate (1:5000) inactivated whole cells and lipopolysaccharide fractions (extracted by alcohol and trichloracetic acid) of B. bronchiseptica. Vaccinated and unvaccinated control mice were then inoculated intraperitoneally with live suspensions of either B. bronchiseptica, B. pertussis,

or B. parapertussis. Eldering (1942) prepared whole cells and lipopolysaccharide fractions of B. pertussis and B. parapertussis, and studied their cross protective ability using the same methodology (Table 1). Cultural and serological data were not reported.

Cross protective ability of the Bordetella species in mice was determined by Kendrick et al. (1953) using intraperitoneal immunization with merthiolate (1:5000) inactivated whole cell vaccines and intracerebral challenge with live cultures. These results were similar to those of Eldering (1941, 1942) regarding mice vaccinated with either B. bronchiseptica, B. pertussis, or B. parapertussis and challenged with B. bronchiseptica. Protection was also adequate with mice vaccinated with B. pertussis or B. bronchiseptica and challenged with homologous culture. Mice vaccinated with either B. bronchiseptica or B. parapertussis did not resist challenge against B. pertussis. The discrepancies in these results may be explained by the variation in the method of challenge and the dose of challenge organisms.

Ross et al. (1969) reported a detailed study of the mouse protective antigens of Bordetella species utilizing the intracerebral challenge test. They found that active immunization with heat inactivated whole cells (56° C for 20 minutes) and a saline extract of B. bronchiseptica protected against homologous challenge but not against challenge by B. pertussis. Whole cells and some saline extracts of B. pertussis protected against both challenge by B. bronchiseptica and B. pertussis.

Table 1. Summary of cross protection studies of the various Bordetella species conducted in mice (Eldering, 1941 and 1942)

Vaccine	Challenge organism		
	B. bronchiseptica	B. pertussis	B. parapertussis
B. bronchiseptica			
Whole cells	7/30[a]	7/25	10/15
Fraction	5/83	7/42	18/34
Unvaccinated	81/85	77/89	45/46
B. pertussis			
Whole cells	21/55	11/32	1/14
Fraction	N.D.[b]	5/29	N.D.
Unvaccinated	30/30	122/138	12/15
B. parapertussis			
Whole cells	1/30	17/27	0/23
Fraction	6/25	27/31	15/42
Unvaccinated	24/27	25/29	54/57

[a]Numerator designates number dead; denominator designates number of mice in group.

[b]Not determined.

Whole cells and a saline extract of B. parapertussis protected against challenge by B. bronchiseptica but not B. pertussis. However, saline extracts of most B. pertussis strains, while protecting against homologous challenge, did not protect against B. bronchiseptica challenge. This suggested that 2 antigens may be responsible for B. pertussis induced protection. The antigen responsible for protection against B. bronchiseptica that was present in saline extracts of B. bronchiseptica was more resistant to heat than the antigen from saline extracts of B. pertussis.

Winsser (1960) attempted active immunization of mice by intraperitoneal injection of whole cells of either B. bronchiseptica or B. pertussis inactivated by merthiolate (1:5000). Both types of vaccinated mice were challenged with B. bronchiseptica intranasally. The B. bronchiseptica vaccinated mice were protected against death and the organism was not recovered from the lungs at necropsy. The B. pertussis vaccinated mice died and B. bronchiseptica was recovered from the lungs at necropsy.

Kendrick et al. (1939) first demonstrated protection against whooping cough in humans by the use of B. pertussis vaccines. Bordetella pertussis was inactivated with merthiolate (1:10,000) or phenol (0.5 percent) and administered subcutaneously to 1815 children. There were 2397 unvaccinated control children in the experiment. Only clinical evaluations were made. There were 52 cases of whooping cough in the vaccinated

group and 348 cases of the disease in the unvaccinated group. The literature contains numerous subsequent reports concerning various vaccine field studies, potency testing of vaccines, purification of the protective antigen of the vaccine, and epidemiological studies involving the carrier state of B. pertussis infection in humans. Only selected references which have relevance to B. bronchiseptica resistance in swine are included in the following discussion.

During the ensuing years since the first report of a successful vaccine for the protection of children against whooping cough, several laboratories have attempted to standardize their vaccines by active or passive immunization of mice followed by challenge by the intraperitoneal or intranasal route. These attempts met with limited success. Kendrick et al. (1947) investigated the possibility of potency testing of vaccines by active immunization of mice followed by challenge by the intracerebral route. This technique was reproducible as conducted by several researchers and detected differences in various vaccines submitted from 10 laboratories. In 1956, the Medical Research Council (of England) published the compilation of a series of studies regarding the potency of various vaccines as compared by field results and intracerebral mouse protection tests. The field and laboratory results showed a correlation between the potency of vaccines in protecting children, their ability to protect mice against intracerebral challenge, and

their ability to produce agglutinin in the serum of both mice and children (Medical Research Council, 1956). Pillemer et al. (1954) attempted to isolate the protective antigen of B. pertussis by sonic disintegration of the organism followed by adsorption with red cell stromata. Efficiency of separation was based on intracerebral mouse protection tests. Evans and Perkins (1955) demonstrated that this purified protective antigen possessed a small quantity of agglutinogen and produced a poor agglutinin response in mice. Recently, Holt and Spasojevic (1968) reported that growth of various strains of B. pertussis on different media would lead to the loss of surface agglutinogens. When these agglutinogen deficient preparations were used as vaccines, they were found by means of intracerebral mouse protection tests to be as protective as parent strains possessing the normal agglutinogen content.

Keogh et al. (1947) reported that whole B. bronchiseptica, B. pertussis, and B. parapertussis cells, filtrates, and supernatants of broth cultures would cause agglutination of human, mouse, and fowl erythrocytes. Keogh and North (1948) demonstrated that human and rabbit antisera possessing high titer anti-hemagglutinin would protect mice against death due to B. pertussis inoculated intranasally. However, a purified hemagglutinin of B. pertussis, prepared by Masry (1952), did not prevent death in mice challenged either by the intracerebral or intranasal routes. Protection was attempted either by active immunization with the hemagglutinin or by passive immunization

with anti-hemagglutinin.

Since it was shown that agglutinin and anti-hemagglutinin were not involved in induction of protection in mice against death due to massive B. pertussis challenge, great effort has been extended in an attempt to isolate, purify, and identify the protective antigen of B. pertussis active in the intracerebral mouse protection test. The extraction method using red cell stromata (Pillemer et al., 1954) was difficult to reproduce with different strains and loss of activity occurred during sonic disruption. Barta (1963) extracted the protective antigen from B. pertussis with sodium desoxycholate. Munoz (1963) found this procedure difficult to reproduce and that the protective antigen extracted by this method contained material which sensitized certain strains of mice to histamine. Munoz and Hestekin (1963) prepared the protective antigen of B. pertussis by saline extraction of fractionated cells. This saline extract was purified by starch block electrophoresis but the protective antigen and histamine sensitizing factor were inseparable and believed to be identical. By contrast, Nagel (1967) separated the protective antigen from the histamine sensitizing factor of B. pertussis by ultracentrifugation.

Although the most prevalent method of studying pertussis immunization has been the use of the intracerebral mouse protection test, several workers have utilized the mouse respiratory tract as a more natural model for investigating resistance

to B. pertussis infection. Cooper (1952) induced resistance in mice against intranasal challenge by B. pertussis by the use of pertussis vaccine administered intranasally. These intranasally immunized mice were not resistant to intracerebral challenge. Cooper also demonstrated that in mice infected with sub-lethal doses of B. pertussis by intranasal inoculation, the number of organisms in the lungs increased to a maximum on the seventh day and declined thereafter. These mice resisted intracerebral challenge at 4 weeks but not 6 weeks after the initial infection. Andersen (1953) inoculated mice intranasally with the sub-lethal doses of B. pertussis and demonstrated the organism in the lungs up to 59 days after exposure. When these mice were challenged intranasally with sub-lethal doses at 70 days after exposure, the lungs were clear of the organism 4-8 days after reinfection. Serum agglutinins were detected 38 days after the first exposure.

Standfast (1958) demonstrated that the potency of vaccines assayed by intranasal challenge was different from the potency assayed by the intracerebral route. He showed that the antigen responsible for intracerebral protection was more heat labile than the antigen responsible for intranasal protection. Dolby and Standfast (1958) found 2 distinct antibodies in rabbit antisera against B. pertussis. One antibody passively protected mice against lethal infection by the intracerebral route and the other passively protected mice against lethal infection by the intranasal route. Andersen and Bentzon (1958) utilized

the number of organisms recovered from the lungs to measure protection of vaccinated mice against challenge with sub-lethal doses of B. pertussis administered intranasally and observed a correlation with vaccines assayed by lethal intracerebral challenge. Dolby et al. (1961) inoculated untreated and actively immunized mice intranasally with lethal doses of B. pertussis. By the 25th day of infection, all control mice had died and all lungs were positive for the organism. Of the vaccinated mice, 5 of 60 died during the 28 day observation period. One vaccinated mouse was necropsied every 5 days and the number of sterile lungs was 60 percent by the end of the experiment.

Dolby and Dolby (1969) prepared rabbit antisera against B. pertussis and fractionated the sera into 7s and 19s globulin components. These components were incubated with lethal doses of the organism before intracerebral challenge and with sub-lethal doses of the organism before intranasal challenge in mice. Evaluation of protection was based on brain and lung counts of B. pertussis. The 7s globulin was 100 times more effective than 19s both in the bactericidal reaction in vitro and in protection against intracerebral challenge. By contrast, the minimal weights of 7s and 19s globulins required to neutralize hemagglutinin and to protect against intranasal challenge were similar. These workers postulated that the antibody responsible for protection in the intracerebral mouse

protection test was bactericidal and differed from the antibody responsible for protection against intranasal infection.

Respiratory Tract Resistance to Infectious Agents

Resistance to infectious agents by the respiratory tract of a mammalian host has been divided into 2 broad categories; these are natural and acquired resistance.

Natural resistance

The respiratory tract has the capacity for the elimination of infectious agent laden particulate matter. Due to the tortuous design of the nasal cavity, most of the air stream during inspiration is concentrated over a small portion of the nasal surface. While during expiration, the air stream is diffusely spread through the entire nasal passage. Malformations such as septal deviations and nasal polyps in humans may concentrate air flow onto an even smaller area which may have a drying effect on the mucosa (Proctor, 1966).

The mucous membrane of the upper respiratory tract of mammals consists of ciliated pseudostratified columnar epithelium beginning at the posterior edge of the anterior end of the nasal turbinates and extending into the nasopharynx (Copenhaver and Johnson, 1958). The nasopharynx contains transitional and stratified squamous epithelium. The trachea and bronchi contain ciliated pseudostratified columnar

epithelium which changes to a nonciliated columnar cell type in the terminal bronchioles. Goblet cells and mucosal glands supply mucus which covers the ciliated portions of the respiratory tract.

The mucus covering the cilia is kept continually in motion by the cilia, traveling posteriorly in the nasal cavity and anteriorly in the tracheobronchial tree to the unciliated portions of the hypopharynx where the mucus is swallowed (Proctor, 1966). Quinlan et al. (1969) demonstrated that small radioactively tagged particles travel at a rate of 7 mm per minute in the nasal mucociliary flow of normal human subjects.

Wells and Wells (1936) reported that microorganisms are usually contained in the inspired air in nuclei that are 2 to 3 microns in diameter. Wells (1955) found that when tubercle bacilli were contained in droplet nuclei less than 3 microns in diameter, a more severe pneumonia resulted in rabbits than if the droplet nuclei were 10-12 microns in size.

Apparently, particulate matter in excess of 10 microns in diameter is readily removed by the mucociliary system due to impingement of these particles in the mucus. Many particles less than 10 microns in size may penetrate the lower respiratory tract to the aveoli of the lung. These particles which penetrate beyond the mucociliary apparatus have been reported to be engulfed and destroyed by the alveolar macrophages. These macrophages may be removed by the mucociliary apparatus or by the lymphatic system (Wright, 1961). When the alveolar macro-

phages or the mucociliary apparatus fails to remove an infectious agent, inflammation of the respiratory tract may occur leading to the influx of polymorphonuclear (PMN) leucocytes (Green, 1968). The PMN leucocytes are also capable of engulfing and destroying microorganisms as a secondary line of defense in the respiratory tract (Weiser et al., 1969).

Weiser et al. (1969) states that the PMN leucocytes have a relatively short duration of life and the disintegrating PMN cells supply hydrolytic enzymes for the destruction of microbial agents. Zeya and Spitznagel (1966) reported that the cationic fraction of lysosomes extracted from PMN cells contained enzymes (including lysozyme) which were bactericidal for streptococci and staphylococci. Hirsch (1956) had previously reported that rabbit PMN leucocytes contained a heat stable protein called phagocytin which was bactericidal for certain Gram negative bacteria. Dajani and Ayoub (1969) demonstrated that a substance from PMN cells would lyse Mycoplasma pneumoniae.

Two types of mucus (sulphomucin and sialomucin) have been reported to be produced in rat respiratory tract secretions (Lamb and Reid, 1968). Viral agents (Luria and Darnell, 1967) and Mycoplasma pneumoniae (Sobeslavsky et al., 1968) have been reported to attach to neuraminic acid receptor sites of tracheal epithelial cells.

Acquired resistance

It is convenient to divide acquired resistance of the respiratory tract into humoral and cellular factors (Austen and Cohn, 1963).

Humoral factors Specific antibody and interferon have been shown to exist in the respiratory secretions of man. Bellanti et al. (1965) reported that virus neutralizing activity of human serum was associated with immunoglobulin (Ig) G while in nasal secretions the antibody activity was associated with IgA. These workers also reported that IgA was the predominant immunoglobulin in nasal secretions. Recovery from respiratory infections caused by parainfluenza Type 1 virus has been correlated with the presence of neutralizing antibodies in nasal secretions rather than in the serum (Smith et al., 1966).

Resistance to A2 influenza virus in humans has been correlated with IgA present in nasal secretions. The IgA was reported to have virus neutralizing and hemagglutination-inhibiting activity (Alford et al., 1967). Bellanti et al. (1967) demonstrated that either injection of an attenuated tularemia vaccine subcutaneously or experimental intranasal infection with Francisella tularensis in humans would induce indirect hemagglutinating antibodies in both the nasal secretions and serum. Resistance was not induced with the vaccine. The serum antibody activity was associated with IgM while the

nasal secretion antibody activity was associated with IgA. No mechanism has been described which would explain the mode of action of IgA in respiratory tract resistance to bacterial infections.

Butler et al. (1967) has shown that 2 types of IgA are present in nasal secretions; these occur as a 7s and an 11s molecule. The 7s IgA molecule has been shown to be present in serum and to originate from serum when present in nasal secretions. The 11s IgA molecule, however, has been demonstrated to be synthesized de novo in the tissues of the upper respiratory tract. Tomasi (1969) reported that the 11s IgA molecule, with a molecular weight of 380,000, consists of a dimer of 7s IgA plus a secretory piece (SP) with a molecular weight of 58,000. The SP has been shown to originate from the respiratory tract epithelium.

Isaacs (1963) reported that production of interferon in the lungs of mice infected with sublethal doses of influenza correlates with the decline of disease symptoms. He also reported that aging mice develop an increased ability to eliminate this virus from their lungs which is accompanied by an increased production of interferon.

Cellular factors Weiser et al. (1969) did not consider PMN leucocytes to be a part of cellular immunity or resistance. These cells do not undergo specific immunological activation upon the 2nd encounter with an infectious agent. The lung

alveolar macrophage represents the principal effector cell type in acquired cellular resistance to respiratory disease.

Dannenberg (1968) has demonstrated that delayed cellular hypersensitivity in tuberculosis is a specific immunological process in which most macrophages and lymphocytes of the host become sensitive to tuberculin. This hypersensitivity is both local and systemic in nature. In cellular immunity or resistance, the activated macrophage is able to destroy tubercle bacilli in its cytoplasm. Cellular immunity in contrast to delayed hypersensitivity is more local in nature and is not a specific immunological process. This lack of specificity is demonstrated by the fact that macrophages with acquired cellular resistance to tuberculosis have an increased ability to destroy other infectious agents such as Brucella melitensis (Elberg et al., 1957), Salmonella spp. and Escherichia coli (Jenkin and Benacerraf , 1960).

Cellular resistance to other respiratory pathogens has been reported; these are Francisella tularensis in the rabbit (Nutter and Myrvik, 1966) and B. pertussis in the mouse (Gray and Cheers, 1967).

MATERIALS AND METHODS

Source of Bordetella bronchiseptica and Bordetella pertussis Isolates

Two isolates of B. bronchiseptica of swine origin were used: (1) virulent strain B was isolated from a pig with turbinate hypoplasia and had been used to produce turbinate hypoplasia in experimental swine (Ross et al., 1963a and Harris and Switzer, 1968)[1], and (2) strain IVDL 374 was isolated from a pig with turbinate hypoplasia which was submitted to the Iowa Veterinary Medical Diagnostic Laboratory, Ames, Iowa[2]. An isolate of B. bronchiseptica designated low-virulence strain D-1 was isolated from a puppy infected with canine distemper[2]. The low-virulence D-1 strain isolate had been shown in a previous study to be incapable of causing turbinate hypoplasia in swine (Ross et al., 1967).

An isolate of B. pertussis, strain 3779 A, was obtained from a commercial biological company where it was used for the preparation of a vaccine for the prevention of whooping cough in humans[3].

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[1]Dr. W. P. Switzer, Veterinary Medical Research Institute, Iowa State University, recovered this isolate.

[2]Dr. R. F. Ross, Veterinary Medical Research Institute, Iowa State University, recovered these isolates.

[3]Eli Lilly and Company, Indianapolis, Indiana.

Source of Pasteurella multocida and Haemophilus spp. Isolates

Pasteurella multocida, serotype D, was isolated from a pig with turbinate hypoplasia submitted to the Veterinary Medical Research Institute, Ames, Iowa. This isolate had been used in combination with B. bronchiseptica, strain B, to produce turbinate hypoplasia in swine (Harris and Switzer, 1968).

Haemophilus spp. was isolated from a pig submitted to the Veterinary Medical Research Institute, Ames, Iowa.

Culture Media

Bordetella bronchiseptica was isolated by inoculating specimens on modified MacConkey's agar[1] containing 1 percent dextrose (Ross, 1963). Pasteurella multocida and Haemophilus spp. were isolated on horse blood agar composed of 5 percent difibrinated horse blood in tryptose blood agar base medium[1].

Bordetella bronchiseptica and P. multocida were propagated in tryptose phosphate broth (TPB)[1]. Media for the biochemical identification of these organisms were commercial dehydrated media[1]. Bordetella pertussis was propagated on horse blood agar prepared by the addition of 16 percent horse blood to Bordet Gengou agar base[1]. Haemophilus spp. was propagated on mycoplasma medium containing fresh beef heart infusion broth, 0.2 percent hemoglobin, 0.5 percent swine gastric mucin, 20

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[1]Difco Laboratories, Incorporated, Detroit, Michigan.

percent turkey serum, and 1.5 percent Noble agar[1] (Ross and Switzer, 1963).

Bordetella bronchiseptica Inocula

Cultures of B. bronchiseptica used in transmission experiments were stored lyophilized in low passage. Inocula of strain B and strain 374 for intranasal and intratracheal inoculations were prepared by injecting 0.1 ml of 24-hour broth cultures into the yolk sac of 6- to 8-day-old embryonating chicken eggs. The embryos were incubated at 37°C and candled daily. Embryos dying after 24 hours incubation were incubated an additional 24 hours. Yolk sac fluids were harvested, checked for purity on 5 percent horse blood agar, and stored frozen at -20°C.

Inocula of D-1 strain usually consisted of 24 hour culture grown in TPB varying between 7 to 12 passages. When D-1 strain was grown in embryonating chicken eggs, it had been passaged less than 6 times in TPB. B strain inocula refers to yolk-sac fluids, and D-1 strain inocula refers to broth culture unless stated otherwise in the results section.

The egg fluid inocula containing B. bronchiseptica strain B had 5.0×10^7 to 1.0×10^9 colony-forming units per ml. The egg fluid inocula containing B. bronchiseptica strain IVDL 374

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[1]Difco Laboratories, Incorporated, Detroit, Michigan.

had approximately 2.0 x 10^6 colony-forming units per ml of the organism. Passage of B. bronchiseptica through embryonating chicken eggs resulted in an increased hemolytic activity on horse blood agar.

The B. bronchiseptica D-1 strain inocula used in experiment X was previously passaged for 21 times on either modified MacConkey's agar or TPB. Inoculum for each passage was the smallest colony with the largest central portion observable on the plate. After 21 passages, the organism appeared on modified MacConkey's agar as a small colony measuring approximately 2 mm in diameter containing a large central elevation. This was about one-half to two-thirds normal size although the central area was larger than normal. Passage 22 was grown in TPB and was used as inoculum.

Respiratory Tract Inoculations

Swine were inoculated intranasally by inserting the tip of a 5 ml syringe (without needle) into the naris. Inoculum (0.5 ml) was injected into each side of the nasal cavity. For intratracheal inoculation, swine were held in dorsal recumbency with their necks extended. The posterior border of the larynx was palpated and fixed in position with the thumb and fore-finger. An area over the trachea was disinfected with 70 per-cent alcohol and a 20-gauge needle attached to an empty 5-ml syringe was then inserted into the trachea. After the needle

had entered the trachea (tracheal air could be easily aspirated), a 5-ml syringe containing 0.5 ml of inoculum was attached to the needle, and the contents were injected.

Identification of Bordetella bronchiseptica, Pasteurella multocida, and Haemophilus spp.

Bordetella bronchiseptica appeared on modified MacConkey's agar as grayish tan colonies after 48 hours of incubation at 37° C. Further identification was based on lack of acid production in dextrose and lactose, hydrolysis of urea within 24 hours, utilization of citrate and alkalization of litmus milk.

Pasteurella multocida appeared as grayish white mucoid colonies on 5 percent horse blood agar after 24 hours of incubation at 37° C. Identification was based on production of acid but not gas from the dextrose, production of indole, and failure to produce acid from lactose.

Haemophilus spp. were identified as minute, flattened colonies of Gram negative organisms satelliting along a Micrococcus sp. streak growth on 5 percent horse blood agar after 24 hours of incubation at 37° C.

Preparation of Buffered Solutions

Phosphate buffered saline (PBS)

The PBS was composed of the following:

Na_2HPO_4 (0.1 M)	85.7 ml
KH_2PO_4 (0.1 M)	14.3 ml

Saline (0.85 percent NaCl)	900.0 ml

The above solution was pH 7.4.

Veronal buffered diluent (VBD)

Stock solution was composed of the following:

NaCl	83.00 gm
Na-5,5-diethyl barbiturate	10.19 gm
Distilled H_2O	1867.23 ml
HCl (1 N)	34.58 ml
Solution containing $MgCl_2$ (1 M) and $CaCl_2$ (0.3 M)	5.00 ml

The VBD was composed of the following:

Stock solution	100.00 ml
Distilled H_2O	400.00 ml
Gelatin (0.25 percent)	16.00 ml

The VBD was pH 7.4 and stored at 4-8° C. It was used within 24 hours of preparation.

Preparation of Vaccines

Sonicate-vaccine

Five ml of a 24-hour broth culture of 6th passage B. bronchiseptica strain D-1 was inoculated onto tryptose blood agar (without blood) in Roux flasks and incubated at 37° C for 48 hours without inversion of the flasks. The growth was harvested in 20 ml of distilled water per flask and frozen at -20° C.

Glass beads were used to loosen the growth from the agar. This material was thawed and centrifuged at 4° C for 30 minutes at 4000 xg. The supernatant was removed and an equal volume of PBS was added. The organisms were re-suspended and 20 ml of the suspension was placed in a Rosett Cooling Cell[1], Model 24, which was contained in a crushed-ice and salt water bath maintained at a temperature of 2-6° C. This suspension was sonicated for 6-3 minute intervals. Sonication was stopped every 3 minutes to allow a 2 minute cooling of the suspension. The sonication was performed with a 12 mm diameter Bronwill Biosonik II[2] ultrasonic probe inserted approximately 4 mm into the suspension. The first sonication interval was initiated at 60 percent of full power. Each succeeding interval was increased until 95 to 100 percent of full power was attained on the final 2-3 minute intervals. Severe foaming of the suspension occurred if 100 percent power was attempted during the initial sonication intervals.

Sonicated material was sterilized by passage through a 0.45 micron APD cellulose filter[3], and the filtrate was frozen at -20° C. On the day of use the filtrate was thawed and emulsified with equal parts Freund incomplete adjuvant by

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[1]Heat Systems Company, Melville, New York.

[2]Bronwill Scientific, Rochester, New York.

[3]Millipore Filter Corporation, Bedford, Massachusetts.

repeated passage through a 20-gauge needle attached to a 10-ml syringe. The mixture (sonicate-vaccine) was injected subcutaneously in the axillary space of young experimental swine in 2 ml amounts in active immunization studies. Sonicate-vaccine was injected subcutaneously in the fold of the flank in pregnant sows in 3 ml amounts for passive immunization studies.

Before sonication, the suspension in PBS contained approximately 2.1 x 10^{10} colony-forming units per ml of B. bronchiseptica or a reading of 12 to 13 nephelos units at 1:1000 dilution of the suspension on a Nepho-Colorimeter[1], adjusted to a standard of 20. After the 6th sonication interval, the suspension contained approximately 1.9 x 10^{8} colony-forming units per ml or a reading of 4 nephelos units at a 1:1000 dilution adjusted to a standard of 20.

Pertussis-vaccine

Pertussis Vaccine Fluid, U.S.P.[2] was emulsified with equal parts of Freund incomplete adjuvant by repeated passage through a 20-gauge needle attached to a 10-ml syringe. The mixture (pertussis-vaccine) was injected subcutaneously in the axillary space of experimental swine in 2 ml amounts of active immunization studies.

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[1]Coleman Instruments Corporation, Maywood, Illinois.

[2]Eli Lilly and Company, Indianapolis, Indiana.

Whole cell-vaccines

Five ml of a 24-hour broth culture of 7th passage B. bronchiseptica strain B was inoculated onto tryptose blood agar (without blood) in Roux flasks and incubated at 37° C for 48 hours without inversion of the flasks. The growth was harvested with the aid of glass beads in 50 ml of 0.85 percent NaCl solution per flask. Formalin was added to the suspension to obtain a final concentration of 1:1000. This suspension was kept at ambient room temperature until it was inactivated and then stored at 4-8° C. Inactivation occurred within 48 hours. This material (whole cell-vaccine, B strain) was injected intramuscularly into the hams of swine in 3 ml amounts.

Five ml of a 24-hour broth culture of 7^{th} passage B. bronchiseptica strain D-1 was inoculated onto Bordet Gengou agar (without blood) in Roux flasks, and the flasks allowed to stand without inversion for 30 minutes at room temperature. The flasks were inverted and incubated for 48 hours at 37° C. Before harvesting, the excess inoculum in the flasks were poured off. Ten ml of 0.85 percent NaCl containing 1:1000 formalin was added to each flask and allowed to stand at room temperature for 1 hour. Glass beads were added and the suspension harvested. Five ml of 0.85 percent NaCl containing 1:1000 formalin was added to each flask for a second harvesting. The harvest from each flask was pooled and refrigerated at 4-8° C.

The organisms were inactivated after 10 days of refrigeration and were used for injection into swine 13 and 21 days after harvest. A 1:100 dilution of the material gave a reading of 71 nephelos units, adjusted to a standard of 20, on a Nepho-colorimeter. The concentrated material was mixed with equal parts Freund incomplete adjuvant by repeated passage through a 20-gauge needle attached to a 10-ml syringe. This mixture (whole cell-vaccine, D-1 strain) was injected subcutaneously into the axillary space of swine in 2 ml amounts.

Source and Care of Experimental Swine

Swine were obtained from the respiratory-disease-free herd maintained at the Veterinary Medical Research Institute, Ames, Iowa. Original stock was surgically derived 19 years ago, and strict health management practices have been conducted since that time. The ration fed both the herd and the experimental pigs did not contain antibiotics, sulfonamides, or arsenicals. The feed contained a calcium:phosphorus ratio satisfying recommendations of the National Research Council (1964).

Three age groups of pigs were used: (1) Newborn pigs were farrowed naturally in isolation units. Each pig was injected intramuscularly with 1 ml of Armidexan[1] (iron dextran solution) at 3 days of age. The pigs had access to a creep ration (18

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[1]Bradley Products Company, Bradley, Illinois.

percent protein) beginning at 1 week of age and were weaned at approximately 4 weeks of age. A grower ration (16 percent protein) was fed from the 5th week of age. (2) Two- to 3- week-old pigs were weaned in the supply herd and were placed in isolation units. The pigs were initially fed the 18 percent protein creep ration and were shifted to the 16 percent protein grower ration at 4 to 5 weeks of age. A preweaning creep ration was prepared by the addition of 10 percent ferrous sulfate to the 18 percent protein creep ration. Early weaning was facilitated by feeding this iron-rich creep to the pigs from the 2nd day of life until 1 week after weaning. The pigs were then fed the regular 18 percent protein creep ration. Additional supplemental iron was not supplied. (3) Four-week-old or older pigs were weaned in the supply herd and fed the grower ration while on experiment.

Animal Experiments

Principal and infected control swine were housed in isolation during the course of the experiments. Unexposed swine were either housed in isolation units or remained in the supply herd until necropsy.

The procedure followed in the animal experiments was variable with regard to age of experimental swine, time of intranasal inoculations, nasal secretion and blood collection, and necropsy. Therefore, these parameters of methodology are

included in the results section to facilitate interpretation of the experiments.

All serum antibody titers against B. bronchiseptica and B. pertussis which are presented in the results section were determined by the particulate antigen settling test unless stated otherwise.

Collection of Specimens

Nasal secretions were collected in vivo on cotton-tipped wooden applicator sticks (swabs) by inserting the swab approximately three-fourths of the length of the nasal cavity and gently twisting the swab as described by Ross et al. (1963b). The nares were cleaned with cotton soaked with 70 percent alcohol prior to insertion of the swab. Samples of nasal secretions for bacterial culture were collected at necropsy by sectioning the head longitudinally and carefully removing the median septum. A sterile swab was then rubbed on the posterior one-half of the ventral turbinate.

Tracheal secretions and lung were aseptically collected at necropsy. Lung tissue was minced with sterile scissors and ground with TPB in a TenBroeck tissue grinder.

Nasal washings were collected by inserting a rubber female catheter into the nasal cavity of a pig with the snout held downward at a 45° angle. A syringe was attached to the catheter, and a 5 ml quantity of PBS was flushed into the nasal cavity

during expiration. Washings which dripped out of the nares around the catheter were collected in a disposable plastic drink cup. The nares were cleaned with cotton soaked with 70 percent alcohol prior to insertion of the catheter (Figure 1).

Nasal washings were checked for the presence of occult blood using Hemastix[1], homogenized by constant stirring at 4-8° C for 4 hours, centrifuged at 4° C for 30 minutes at 4000 xg. The supernatant was concentrated by the addition of Lyphogel[2] and maintained at 4-8° C overnight. The concentrated nasal washings were frozen at -20° C.

Swine blood samples were collected from the anterior vena cava.

Histological Procedures

Portions of the ventral turbinates, trachea, and lungs were fixed for a minimum of 48 hours in 10 percent buffered formalin. Cross sections of the turbinate, in the area of maximal scroll development, were decalcified by 1 of the following methods: (1) The tissue was placed in Decal[3] solution for 48 hours at room temperature and then washed overnight in tap water before embedding. (2) The tissue was placed in a

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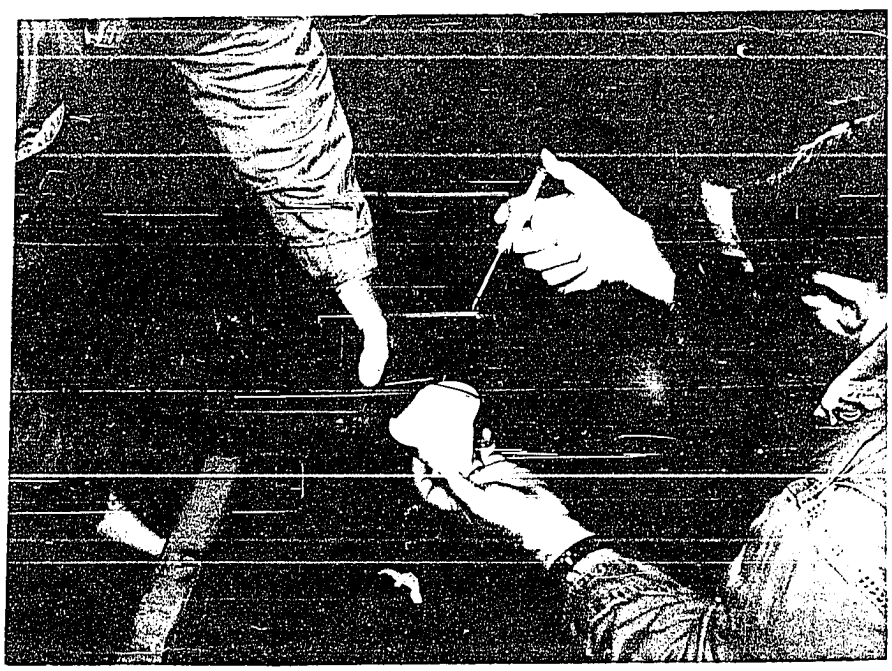
[1]Ames Company, Incorporated, Elkhart, Indiana.

[2]Gelman Instrument Company, Ann Arbor, Michigan.

[3]Scientific Products, Evanston, Illinois.

Figure 1. Nose of a pig was held downward at an approximate 45° angle for collection of nasal washings. A female catheter was inserted into the nasal cavity and 5 ml of sterile PBS was dispensed slowly into each naris. The nasal washings were collected in a disposable drink cup



solution of 3 percent disodium ethylenediamine tetraacetate[1] (EDTA) which was prepared in PBS. The tissues remained in the EDTA for a minimum of 10 days and were then placed in 70 percent ethyl alcohol until the embedding process.

The tissues were embedded in Paraplast[2] tissue embedding medium, sectioned at 6 microns and mounted on glass slides with an albumin fixative. Staining was done with methyl green-pyronine as described by Pearse (1960) or with Harris' hematoxylin and counterstained with eosin Y as described in the U.S. Armed Forces Institute of Pathology (1960) Manual of Histologic and Special Staining Techniques. Decalcification with EDTA solution was required to obtain characteristic staining by methyl green-pyronine.

Antigens

Bordetella bronchiseptica broth antigens

D-1 and B strain (8th passage) were grown in TPB for 16 hours at 37° C and then a 1 ml aliquot was inoculated into 550 ml of TPB in 1000 ml Erlenmeyer flasks. These cultures were incubated 20-24 hours at 37° C. For live broth antigens, the growth was harvested by centrifugation at 5-10° C at 15,000 xg for 1 hour. The sediment was resuspended in 0.85 percent NaCl

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[1]Cambridge Chemical Products, Incorporated, Detroit, Michigan.

[2]Brunswick Laboratories, St. Louis, Missouri.

and stored at 4-8° C. For inactivated broth antigens, formalin was added to the flasks at a 1:1000 concentration and incubation at 37° C was continued for another 20-24 hours. Cultures on blood agar revealed that the organisms were inactivated after this treatment. Cells were harvested by centrifugation at 5-10° C at 15,000 xg for 1 hour, resuspended in 0.85 percent NaCl containing 1:1000 formalin and stored at 4-8° C.

Bordetella bronchiseptica agar antigens

D-1 and B strain (8th passage) were grown in broth for 24 hours at 37° C and then 5 ml aliquots were inoculated into Roux flasks containing Bordet Gengou agar (without blood). The flasks were incubated for 24 hours at 37° C in an inverted position. For live agar antigens, a 0.85 percent NaCl with glass beads was used to suspend the growth. This suspension was stored at 4-8° C. For inactivated agar antigens, glass beads in 0.85 percent NaCl containing 1:1000 formalin were used to suspend the growth. This suspension was incubated at 37° C for 24 hours, and then stored at 4-8° C. The organisms were inactivated after 24 hours incubation at 37° C.

Bordetella pertussis antigen

Strain 3779A was grown on 16 percent horse blood Bordet Gengou agar for 48 hours at 37° C. The growth was harvested with 0.85 percent NaCl with glass beads and stored at 4-8° C.

Salmonella spp. antigens

Salmonella binza and S. virginia[1] were grown in broth at 37° C for 18 to 20 hours and then 5 ml aliquots were transferred to Roux flasks containing tryptose agar (without blood). The flasks were incubated at 37° C for 20-24 hours without inversion. For H antigens, the growth was harvested with 0.85 percent NaCl containing 1:200 formalin. The suspension was incubated overnight at room temperature for inactivation of the organisms and then stored at 4-8° C. For O antigens, growth in flasks was harvested with 0.85 percent NaCl solution. Three volumes of 95 percent ethyl alcohol were added; the suspension was incubated at 37° C for 2 hours and then centrifuged for 30 minutes at 4000 xg at 4-8° C. The sediment was resuspended in 0.85 percent NaCl containing 0.3 percent phenol and stored at 4-8° C.

Antisera

Swine antisera

Sera used in the various serologic tests utilizing B. bronchiseptica antigens were procured from the animal experiments involving swine infected intranasally with live organism or injected with a vaccine containing the organism. Sera

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[1]Obtained from Dr. M. L. Kaeberle, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University.

containing antibody against Salmonella spp. O and H antigens was produced by injecting the appropriate antigen into 4-month-old swine. The antigen, in concentrated form, was mixed with equal parts Freund incomplete adjuvant and injected subcutaneously into the axillary space. Three ml amounts of antigen were injected 2 times at 2 week intervals. Blood was collected approximately 2 weeks after the last injection.

Rabbit antisera

Sera containing antibody to B. bronchiseptica and Salmonella spp.[1] were produced by intravenous injections of various inactivated suspensions of the organisms adjusted to a tube 3 McFarland nephelometer[2]. The following injection schedule was followed: day 1, 0.5 ml; day 4, 1.0 ml; day 8, 2.0 ml; day 12, 3.0 ml; and day 16, 3.0 ml. The rabbits were anesthetized with barbiturates and bled by heart puncture on day 21. The Salmonella spp. O and H antigens and the B. bronchiseptica inactivated agar antigens were used for production of the antisera.

Identification of antisera sample numbers

The source of swine and rabbit sera which were used in various serologic tests are listed in Tables 2 and 3. The

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[1]Salmonella spp. antisera produced and supplied by the students participating in Veterinary Microbiology and Preventive Medicine, Serology 520, fall quarter, 1969.

[2]Nephelometer prepared according to procedure in Kolmer and Boerner (1931).

Table 2. Source of serum samples from swine

Sample no.	Experiment no.	Day of experiment	No. of donor swine	Exposure[a] and/or vaccination Experience of donor swine
1	XVI	57	4	whole cell-vaccine (B strain) - exposed to B strain
142	XIII	105	1	sonicate-vaccine - exposed to B strain
383a	VIII	43	3	D-1 strain cleared - unexposed to B strain
422	XIV	3	12	unexposed control - 3 days old
424a	XVII	40	4	whole cell-vaccine (D-1 strain) - unexposed to B strain
460a	XIV	21	4	sonicate-vaccine - unexposed to B strain
466a	XIV	21	4	pertussis-vaccine - unexposed to B strain
530	VIII	106	2	D-1 strain cleared - exposed to B strain
543	XIII	96	4	pertussis-vaccine - exposed to B strain
544	XIII	96	2	sonicate-vaccine - exposed to B strain
545	XIII	96	3	unvaccinated - exposed to B strain

[a]Intranasal inoculation with B. bronchiseptica (virulent B strain or low-virulence D-1 strain).

Table 2 (Continued)

Sample no.	Experiment no.	Day of experiment	No. of donor swine	Exposure[a] and/or vaccination Experience of donor swine
572	--	--	2	unexposed control - 6-8 weeks old
573	--	--	1	pre-injection bleed for no. 614
574	--	--	1	pre-injection bleed for no. 615
575	XVII	100	4	whole cell-vaccine (D-1 strain) - exposed to B strain
576	VIII	131	4	D-1 strain cleared - exposed to B strain
614	--	--	1	injected S. binza H antigen
615	--	--	1	injected S. binza O antigen
685	XIV	176	2	pertussis-vaccine - exposed to B strain
686	XIV	176	2	sonicate-vaccine - exposed to B strain
691	XIV	176	4	unvaccinated - exposed to B strain
693	--	--	2	unexposed control - 6-8 weeks old
694	--	--	2	unexposed control - 6-8 weeks old

Table 3. Source of individual serum samples from rabbits

Sample no.	Route of inoculation	Antigen preparation injected
33	Intravenous	S. virginia H antigen
34	Intravenous	S. virginia O antigen
37	Intravenous	S. binza H antigen
38	Intravenous	S. binza O antigen
NRS	Normal rabbit serum[a]	None
591	Pre-injection for sample 633	None
592	Pre-injection for sample 634	None
633	Subcutaneous	B. bronchiseptica (D-1 strain) inactivated agar antigen
634	Subcutaneous	B. bronchiseptica (B strain) inactivated agar antigen

[a]No history of exposure to Salmonella spp.

source of nasal washings collected from swine is presented in Table 4.

Serologic Tests

Particulate antigen settling test (PAST)

The test was performed in plastic trays[1] with U-shaped wells. Diluent for antisera dilutions with B. bronchiseptica

[1]Limbro Chemical Company, New Haven, Connecticut.

Table 4. Source of pooled nasal wash samples from swine

Sample no.	Experiment	Day of experiment	No. of donor swine	Hemoglobin[a]	Concentration[b]	Exposure[c] and/or vaccination experience of donor swine
308	V	56	8	-	5X	D-1 strain cleared-unexposed to B strain
309	VII	62	8	-	5X	D-1 strain cleared-unexposed to B strain
310	VII	74	8	-	10X	D-1 strain cleared-unexposed to B strain
379	XIII	96	3	+	2X	exposed to B strain
380	XIII	96	4	+	2X	pertussis-vaccine - exposed to B strain
381	XIII	96	2	±	2X	sonicate-vaccine - exposed to B strain
435	VIII	67	2	-	2X	exposed to B strain
436	VIII	67	4	-	3X	D-1 strain cleared - exposed to B strain
437	VIII	68	2	-	3X	exposed to B strain
438	VIII	68	4	±	3X	D-1 strain cleared - exposed to B strain
442	XIV	13	8	-	4X	sonicate-vaccine - unexposed to B strain

[a]Determined by an occult blood test.

[b]Indicates the reduction in volume due to concentration.

[c]Intranasal inoculation with B. bronchiseptica either virulent B strain or low-virulence D-1 strain.

Table 4 (Continued)

Sample no.	Experiment	Day of experiment	No. of donor swine	Hemoglobin[a]	Concentration[b]	Exposure[c] and/or vaccination experience of donor swine
443	XIV	13	4	-	3X	pertussis-vaccine - unexposed to B strain
456	VIII	74	2	-	2X	exposed to B strain
457	VIII	74	4	-	3X	D-1 strain cleared - exposed to B strain
472	XIV	21	8	±	6X	sonicate-vaccine - unexposed to B strain
473	XIV	21	4	-	10X	pertussis-vaccine - unexposed to B strain
474	VIII	78	2	-	4X	exposed to B strain
475	VIII	81	4	±	6X	D-1 strain cleared - exposed to B strain
489	VIII	81	4	-	5X	D-1 strain cleared - exposed to B strain
493	VIII	83	4	-	3X	D-1 strain cleared - exposed to B strain
499	--	--	4	-	4X	unexposed control - 8-week-old
500	VIII	95	4	-	5X	D-1 strain cleared - exposed to B strain
533	--	--	6	-	5X	unexposed control - 8-week-old
534	VIII	113	4	-	5X	D-1 strain cleared - exposed to B strain
652	VII	90	8	-	3X	D-1 strain cleared - unexposed to B strain
653	VII	90	4	-	3X	unexposed control

and B. pertussis antigens was 5.0 percent NaCl while diluent for antisera dilutions with Salmonella spp. antigens was 2.5 percent NaCl. Diluent was added to all the wells in 0.05 ml amounts with a dropper[1] and 0.05 ml of serum was added to the first well. Dilutions were made with a diluter[1] which carried 0.05 ml of fluid from one well to the next to give 2-fold dilutions.

Five one-hundredth ml of the appropriate antigen dilution was added to each well. The plate was then covered with a plastic sealer, shaken for 2 minutes on a shaker apparatus[2], and incubated at 37° C for 2 hours. The trays were placed at 4-8° C for 20 to 40 hours. The results were read, following incubation, with the aid of a mirror and recorded. The length of time for incubation at 4-8° C was determined by the presence of buttons in the control wells containing 0.85 percent NaCl instead of antisera.

The proper antigen dilution was determined by block titrations with several sera from different sources. The B. bronchiseptica antigen was diluted in 0.85 percent NaCl to 50 nephelos units on a Nepho-Colorimeter at a standard of 79 at an expanded scale of 4x. This dilution of antigen contained approximately 8.7×10^8 colony forming units per ml and was less than a tube 1 McFarland nephelometer in opacity.

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[1]Cooke Engineering Company, Alexandria, Virginia.

[2]Arthur H. Thomas Company, Philadelphia, Pennsylvania.

The B. pertussis antigen was diluted in 0.85 percent NaCl to a tube 4 McFarland nephelometer which contained approximately 1.78×10^8 colony forming units per ml. Bordetella bronchiseptica and B. pertussis live antigens were utilized in the test within 48 hours of harvest.

The Salmonella spp. antigens were diluted in 0.85 percent NaCl to a tube 2 McFarland nephelometer.

Microagglutination test (MAT)

This test was performed by the same method as the PAST. Results for the PAST were recorded, the trays remained at room temperature for 1 to 2 hours and then were shaken for 10 minutes on a shaker apparatus. The plastic sealer was removed and the contents of each well was observed through a dissecting microscope at 28x magnification for evidence of agglutination.

Tube agglutination test

The test was performed in 8 x 75 mm glass tubes. Diluent with B. bronchiseptica antigens was 5.0 percent NaCl while diluent for Salmonella spp. antigens was 2.5 percent NaCl. Two-fold dilutions were conducted with each sera with individual plastic serological pipettes. Each dilution of the sera and a 0.85 percent NaCl control was pipetted back into tubes in 0.5 ml amounts.

Five tenths ml of the appropriate antigen dilution was added to each tube. The rack of tubes was placed in a plastic bag, sealed, and incubated for 2 hours at 37° C followed by

20-24 hours at 4-8° C. The results were read, following incubation, by visual inspection of tubes shaken by hand. Endpoints were based on the last tube to show clumping of the antigen.

The proper antigen dilution was determined by block titration. The B. bronchiseptica antigen was diluted in 0.85 percent NaCl to 110 nephelos units on a Nepho-Colorimeter adjusted to a standard of 79 at an expanded scale of 4x. The Salmonella spp. antigens were diluted in 0.85 percent NaCl to a tube 2 McFarland nephelometer.

Plate agglutination test

The test was performed by mixing 0.05 ml of antigen and 0.05 ml of diluted serum, gently rotating the glass plate at 2-minute intervals, and examining the antigen-serum mixture for agglutination at the end of 8 minutes. Endpoints were based on the last dilution to show clumping of the antigen. The dilution of the serum was conducted as in the tube agglutination procedure.

The proper antigen dilution was determined by block titration. This dilution was too concentrated to measure by comparative nephelometry but was approximately 25x more concentrated than the antigen dilution used in the PAST.

Direct hemagglutination inhibition test (HAI)

Four- to 8-week-old swine from the source herd were bled on the day of the test. The blood was collected aseptically

in heparin and washed 3 times with VBD. Erythrocytes were resuspended at a 1 percent concentration in VBD. Bordetella bronchiseptica strain D-1 grown on 5 percent horse blood agar for 40-48 hours was suspended in PBS containing 0.01 percent cysteine hydrochloride, pH 7.4.

Serial 2-fold dilutions of the D-1 strain suspension were made in sterile screw-capped glass tubes. Five one-hundredth ml of PBS was added to 12 wells of a plastic tray (U-shaped wells) and 0.05 ml of each dilution of the suspension was then added to each of the wells. Twenty-five thousandth ml of 1 percent erythrocyte suspension was added to each well and the tray was covered with a plastic sealer. The tray was shaken for 2 minutes on a shaker apparatus, incubated for 1 hour at 37° C, and read with the aid of a mirror. The reciprocal of the last dilution exhibiting agglutination of the erythrocytes was recorded as the titer of hemagglutinin present in the suspension.

Dilutions of antiserum were made in PBS. This diluent was added in 0.05 ml amounts to all wells except the 1st well in the series. One-tenth ml of serum or nasal wash was added to the 1st well and dilutions were made with a diluter which carried 0.05 ml of fluid from 1 well to the next, giving 2-fold dilutions. Two units of hemagglutinin were added to each well in 0.05 ml of fluid. Twenty-five thousandth ml of 1 percent erythrocytes was added to each well and the tray was covered

with a plastic sealer. The tray was then shaken for 2 minutes on a shaker apparatus, incubated 1 hour at 37° C, and then incubated at 4-8° C for 18 hours. Each tray contained both a positive and a negative control. The results were read with the aid of a mirror and recorded.

Opsonocytophagic test

Bordetella bronchiseptica strains B and D-1 (passage 9) were stored on tryptose blood agar (without blood) slants at 4-8° C. Broth was inoculated from the slants and incubated for 48 hours at 37° C. Broth was added to the D-1 strain growth until the opacity of the tube was similar to the opacity of the growth in tube containing the B strain. A 1:100 dilution of these suspensions registered approximately 10 nephelos units on a Nepho-Colorimeter at a standard of 20. The adjusted broth cultures each contained approximately 1.1×10^9 colony forming units per ml.

Four- to 8-week-old swine from the source herd were bled on the day of the test. The blood was collected aseptically in heparin. The sera (diluted 1:4 in 0.85 percent NaCl) or nasal wash (concentrated) were sterilized by passage through 0.45 micron APD cellulose membrane filters.

The test system consisted of the following placed in sterile screw-capped glass tubes: (1) 0.2 ml of the adjusted broth culture, (2) 0.2 ml of fresh heparinized swine blood, and

(3) 0.2 ml of serum or nasal wash. The tubes were placed in a drum revolving at 3 to 4 RPM and incubated at 37° C for 1 hour. Smears were made on glass slides, air dried, fixed in methyl alcohol for 2 minutes, and stained with 2 percent Giemsa stain for 15 minutes.

One hundred mature polymorphonuclear cells were observed microscopically in each smear. The number of cells containing bacteria (percent phagocytosis) and the number of bacteria in the 100 cells were determined (phagocytic index). The phagocytic index was calculated by dividing the number of bacteria present in 100 cells by 100.

Bactericidal test

Bordetella bronchiseptica strain B (passage 9) was stored on tryptose blood agar base (without blood) slants at 4-8° C. Broth was inoculated from slants and incubated for 18 hours at 37° C. The culture was diluted with broth to a 36 nephelos units reading on a Nepho-Colorimeter at a standard of 20. The complement was obtained from the serum of a 6-week-old pig from the source herd. The sera, nasal washes, and normal swine serum (as a source of complement) were diluted and filter sterilized as previously described.

The test solution consisted of the following placed in sterile screw-capped glass tubes: (1) 0.5 ml of the adjusted broth culture, (2) 0.3 ml of the serum or nasal wash, and (3) 0.2 ml of normal swine serum for complement. The tubes were

placed in a 37° C waterbath for 1 hour. One ml of the test solution was pipetted into glass tubes containing 9.0 ml of sterile 0.85 percent NaCl solution. Serial 10-fold dilutions were made and 1 ml of each dilution was placed in sterile plastic petri dishes. Tryptose blood agar base (without blood), maintained at 50° C, was added to the petri dishes and the dishes rotated by hand for thorough mixing. The agar was allowed to solidify and the dishes were incubated at 37° C for 48 hours. The number of colonies was recorded from the 10^7 dilution of the test solution.

Agglutination of Erythrocytes and Epithelial Cells

Suspensions of B. bronchiseptica, B. pertussis, Haemophilus spp., and P. multocida were grown on agar surfaces, suspended in PBS with 0.01 percent cysteine hydrochloride, washed 3 times in PBS and diluted in serial 2-fold dilutions in PBS. Erythrocytes from various animal species were collected in heparin or citrate anticoagulant, washed 3 times in VBD, and resuspended at a concentration of 1 percent in VBD. Hemagglutinin titrations were performed as previously described in plastic trays with U-shaped wells.

Swine nasal epithelial cells were collected by scraping the mucosa of the turbinates and median septum with a sterile glass microscope slide. The epithelial cells were suspended in VBD and pipetted vigorously to disperse the cells. The

epithelial cells were washed 3 times in VBD and resuspended at a concentration of 5 percent in VBD. Twenty-five one hundredth ml of 0.25 percent sheep or swine erythrocytes, 0.25 ml of epithelial cells, and 0.5 ml of concentrated B. bronchiseptica cells were mixed and incubated for 30 minutes at 37° C. A wet mount preparation of the mixture was examined by light microscopy. A control mixture containing erythrocytes and epithelial cells without B. bronchiseptica cells was also observed microscopically.

Phagocytosis of Bordetella bronchiseptica

These determinations were performed as previously described for the opsonocytophagic test except 0.2 ml of serum or nasal wash was replaced with 0.2 ml of 0.85 percent NaCl in the test solution.

Statistical Analysis

Chi-square and analysis of variance calculations were conducted as described in Snedecor and Cochran (1967).

RESULTS

Swine Experiments

Infection of the swine nasal cavity with virulent Bordetella bronchiseptica (B strain) and clearance by sulfonamide therapy to induce resistance

Experiment I Ten 4-week-old pigs were inoculated intranasally with virulent B. bronchiseptica (B strain) on day 1. On day 9, all swine were culturally positive for the organism and a sulfonamide[1] was administered via the feed (11 Gm./100kg.) to the swine. The sulfonamide therapy was discontinued on day 33 at which time the organism was no longer present in the nasal cavity. On day 40, the 10 principal swine and 5 unexposed control swine of the same age were inoculated once intranasally with virulent B. bronchiseptica (B strain).

On day 47, the nasal secretions of the 10 drug-cleared swine and 2 of the 5 control swine were negative for the organism. On day 67, the nasal secretions of 8 of 10 drug-cleared swine and 1 of 5 control swine were negative for the organism.

Experiment II Four 8-week-old pigs were inoculated intranasally on days 1, 3, and 5 with virulent B. bronchiseptica (B strain). On day 36, all swine were culturally positive for the organism and the serum titers ranged from 8 to 32. A sulfonamide[1] was administered via the feed (11 Gm./100 kg.) to the

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[1]Sulfamethazine, American Cyanamide Company, Princeton, New Jersey.

swine and continued to day 66. On day 66 the organism was no longer present in the nasal secretions, and serum titers of the swine ranged from 4 to 16. The 4 principal swine and 2 unexposed control swine of the same age were inoculated intranasally on days 88, 90, and 92 with virulent B. bronchiseptica (B strain). On day 88, the serum titers of the principal swine ranged from 4 to 8.

Nasal secretions were collected on day 99; three of the 4 principal swine (1 sample was lost) were negative, and the 2 control swine were positive for the organism. Nasal secretions were collected at necropsy on day 123; the 4 principal swine were negative, and the 2 control swine were positive for the organism. At necropsy the serum titers of the principal swine ranged from 8 to 16, and the control swine were both 32. Macroscopic lesions were not observed at necropsy in either principal or control swine. Microscopically, the epithelium of the ventral turbinates of the control swine was hyperplastic and contained microabscesses filled with segmented neutrophils. Epithelial damage was not observed in the turbinates of the principal swine. Both principal and control swine turbinates contained focal accumulations of pyroninophilic mononuclear cells in the lamina propria.

Infection of the swine nasal cavity with low-virulence Bordetella bronchiseptica (D-1 strain) and recovery from infection by natural elimination to induce resistance

Experiment III Four 4-week-old pigs were inoculated intranasally on days 1, 3, and 5 with low-virulence B. bronchiseptica (D-1 strain, egg passage). On day 11, the nasal secretions of 3 of the 4 principal swine were positive for the organism. Nasal secretions collected on day 25 from all 4 swine were negative for the organism. The 4 principal swine and 4 unexposed control swine of the same age were inoculated intranasally on day 32 with virulent B. bronchiseptica (B strain).

Nasal secretions were collected on day 39; the 4 principal swine were negative, and the 4 control swine were positive for the organism. The 4 principal swine and the same 4 control swine were inoculated intranasally on days 51, 52, and 54 with virulent B. bronchiseptica (B strain). Nasal secretions were collected on day 62; the 4 principal swine were negative, and the 4 control swine were positive for the organism. The 4 principal swine were inoculated intranasally on days 69, 70, 71, 72, 73, 74, 75, 76, 77, and 78 with virulent B. bronchiseptica (B strain). Nasal secretions collected from the principal swine on days 72 and 83 were negative for the organism although 1 sample was not interpretable on day 83 due to bacterial overgrowth.

Experiment IV Twelve 6 to 8-week-old pigs were inoculated intranasally on days 1 and 3 with low-virulence B. bronchiseptica (D-1 strain). Nasal secretions were collected on day 6. Eleven of the swine were positive for the organism while 1 sample was not interpretable due to bacterial overgrowth. On day 12, one pig was destroyed because of a rectal prolapse. Nasal secretions were again collected on day 26 at which time only 1 of the 11 swine was positive for the organism. All 11 nasal secretions collected on day 40 were negative for B. bronchiseptica. Three of the principal swine and 4 unexposed control swine of the same age were moved to a separate isolation unit and inoculated intranasally with virulent B. bronchiseptica (B strain) on days 40, 42, and 44. Nasal secretions were collected on day 50; the 3 principal swine were negative, and 2 of the 4 control swine were positive for the organism.

Four additional principal swine and 2 unexposed control swine of the same age were moved to a separate isolation unit and inoculated intranasally with virulent B. bronchiseptica (B strain) on days 95, 97, and 99. Nasal secretions were collected on day 105; the 4 principal swine were negative, and the 2 control swine were positive for the organism.

The 4 remaining principal swine and 2 unexposed control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 125, 127, and 129. Nasal secretions were collected on day 135. The 4 principal swine were negative, and the 2 control swine were positive for the

organism. A summary of the bacteriologic data from Experiment IV is presented in Table 5.

Experiment V Eight 2-week-old pigs were inoculated intranasally on days 1, 3, and 5 with low-virulence B. bronchiseptica (D-1 strain). Nasal secretions were collected on day 12; seven of the swine were positive for the organism while 1 sample was not interpretable due to bacterial overgrowth. On day 37, one pig died as a result of a severe diarrhea. All the nasal secretions of the swine were negative for B. bronchiseptica on day 43.

On day 56, the 7 principal swine and 4 unexposed control swine of the same age were inoculated both intranasally and intratracheally with virulent B. bronchiseptica (B strain). On days 58 and 60, all swine were inoculated intranasally. On day 56, the serum titer of 1 principal pig was 4 while the other 6 principal swine serum titers were negative. On day 63, two principal swine and 1 control swine were necropsied. On day 65, all of the remaining principal and control swine were necropsied. Macroscopic lesions were not present.

A summary of the incidence of B. bronchiseptica recovered from the respiratory tract secretions of the pigs in Experiment V is presented in Table 6. At necropsy the serum titers for 4 of the principal swine were negative. The other 3 had titers of 4, 4, and 16. The serum titers of the 4 control swine were negative, 8, 16, and 32, respectively.

Table 5. Incidence of Bordetella bronchiseptica in nasal secretions of swine recovered from infection with low-virulence D-1 strain and subsequently inoculated with virulent B strain (Experiment IV)

Swine	Number of swine	First day of B. bronchiseptica (B strain) inoculation[a]	Number of swine yielding B. bronchiseptica in nasal secretions[b]
Principals	3	40	0
	4	95	0
	4	125	0
Controls	4	40	2
	2	95	2
	2	125	2

[a]Number of days after principals were first exposed to low-virulence B. bronchiseptica (D-1 strain).

[b]Based on recovery of B. bronchiseptica from samples of nasal secretions collected 10 days after swine were intranasally inoculated with B. bronchiseptica (B strain).

Microscopically, the turbinates of the positive control swine exhibited mild epithelial hyperplasia, and the exudate on the surface of the epithelium contained many neutrophils. The turbinates of the principal swine all contained focal accumulations of mononuclear cells in the lamina propria although the epithelium appeared normal. These accumulations were in greater

Table 6. Incidence of Bordetella bronchiseptica in respiratory tract of swine recovered from infection with low-virulence D-1 strain and subsequently inoculated both intranasally and intratracheally with virulent B strain (Experiment V)

Swine	Number of swine	Day of necropsy[a]	Number of swine yielding B. bronchiseptica in respiratory tract		
			Turbinates	Trachea	Lung
Principals	2	7	0	0	0
	5	9	0	0	0
Controls	1	7	1	1	1
	3	9	3	3	0

[a]Day swine were killed after first intranasal challenge inoculation with virulent B. bronchiseptica (B strain). Principal swine were first exposed to low-virulence B. bronchiseptica (D-1 strain) 56 days prior to challenge by B strain.

numbers in the ventral scroll of the ventral turbinate. Sections of the tracheas and lungs of the control and principal swine were normal.

Experiment VI Eight 2-week-old pigs were inoculated intranasally on days 1, 3, and 5 with low-virulence B. bronchiseptica (D-1 strain). Experiment VI was initiated on day 1 of Experiment V, and the same inoculation culture of B. bronchiseptica (D-1 strain) was utilized. Two principal swine and 1

previously unexposed control swine of the same age were necropsied on days 8, 15, 22, and 29. The bacteriologic recovery and macroscopic lesions observed at necropsy are summarized in Table 7.

Microscopic lesions of the nasal turbinates included epithelial hyperplasia, microabscesses in the epithelium, and cilia loss in the principal swine necropsied on day 8, 15, 22, and 29. The principal swine necropsied on day 15 and 22 had hypoplasia of the turbinate osseous core, microscopically. A diffuse infiltration of mononuclear cells in the turbinate lamina propria of the principal swine was present in those necropsied on day 22 and 29. Microscopically, the lungs of 4 of the principal swine contained neutrophils in the alveoli and bronchioles and mild alveolar wall thickening. The tracheas of the principal swine appeared normal. Lesions were not observed microscopically in the respiratory tract in any of the unexposed control swine.

Experiment VII Eight 2-week-old pigs were inoculated intranasally on days 1, 3, and 5 with low-virulence B. bronchiseptica (D-1 strain). Nasal secretions collected from 4 of the swine were positive for the organism on day 21, while 2 samples were not interpretable due to bacterial overgrowth. All nasal secretions were negative for B. bronchiseptica on day 61. On day 62, the serum titers were negative from 3 of the swine while 4 had titers of 8, 8, 8, and 16 (1 sample was not obtained).

Table 7. Incidence of Bordetella bronchiseptica and macroscopic lesions in respiratory tract of swine infected with low-virulence D-1 strain (Experiment VI)

Group	Day post-infection	B. bronchiseptica Recovered in respiratory secretions			Macroscopic lesions	
		Turbinates	Trachea	Lung	Turbinate hypoplasia	Pneumonia
Principal	8	+	-	-	-	-
Principal	8	+	+	-	-	-
Unexposed control	-	-	-	-	-	-
Principal	15	-	-	-	- (purulent exudate)	-
Principal	15	-	-	-	- (purulent exudate)	-
Unexposed control	-	-	-	-	-	-
Principal	22	+	+	+	+ (unilateral)	-
Principal	22	-	+	-	-	-
Unexposed control	-	-	-	-	-	-
Principal	29	-	-	-	-	-
Principal	29	+	-	-	-	-
Unexposed control	-	-	-	-	-	-

On day 90, the 8 principal swine and 4 unexposed control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain). Nasal washings were collected from 2 principal and 2 control swine at 0.5, 2, 6 and 26 hours postinoculation. These nasal washings were cultured to determine the number of bacteria in both the whole nasal wash and in the supernatant after centrifugation (10 minutes at 65 xg). The number of B. bronchiseptica recovered from the nasal washings collected on day 90 and 91 is presented in Table 8. The sediment from the centrifuged nasal washings contained epithelial cells, neutrophils, and mononuclear cells. The sediments of principal and control swine appeared similar at each interval. Nasal secretions were collected from 2 principal and 2 control swine (different from the swine used for nasal washing collection) at 6 and 26 hour intervals from the time of intranasal inoculation. The nasal secretions of the 2 principal swine were negative; and 1 of the 2 control swine was positive for B. bronchiseptica at 6 and 26 hour postinoculation.

On day 105, one principal pig died probably from the porcine stress syndrome. On day 107, the 7 principal swine and the 4 previously exposed control swine were inoculated intranasally with virulent B. bronchiseptica (B strain). One principal and 1 previously exposed control swine were necropsied between 1 and 2 hours after this intranasal inoculation. Nasal and tracheal washings were collected at necropsy and cultured

Table 8. Incidence of Bordetella bronchiseptica in nasal washings of swine recovered from infection with low-virulence D-1 strain and subsequently inoculated with virulent B strain (Experiment VII, days 90 and 91)

Group	Pig number	Time post-inoculation (hours)	Number of colony-forming units of B. bronchiseptica recovered from the nasal washings per ml.	
			Whole	Supernatant
Principal	83G	0.5	4.16×10^4	2.28×10^4
Principal	61G	0.5	7.6×10^3	8.8×10^3
Control	82B	0.5	3.9×10^4	2.1×10^4
Control	80G	0.5	8.6×10^4	8.0×10^4
Principal	83G	2	0	0
Principal	61G	2	0	0
Control	82B	2	3.8×10^4	3.1×10^4
Control	80G	2	1.3×10^4	4.8×10^3
Principal	83G	6	2×10^0	0
Principal	61G	6	0	0
Control	82B	6	1.6×10^3	0
Control	80G	6	2.4×10^1	1.0×10^1
Principal	83G	26	0	0
Principal	61G	26	0	0
Control	82B	26	2.4×10^3	8.4×10^3
Control	80G	26	4.0×10^2	8.0×10^2

to determine the number of bacteria in both the whole wash and in the supernatant after centrifugation (10 minutes at 65 xg). In the principal swine, the nasal wash did not contain B. bronchiseptica while the tracheal whole wash contained 2 x 10^3 colony-forming units per ml, and the supernatant contained 1.8 x 10^2 colony-forming units per ml of the organism. In the control swine, the whole nasal wash contained 3.2 x 10^3 colony-forming units per ml while the supernatant contained 4.4 x 10^3 colony-forming units per ml. of B. bronchiseptica. Both the whole and supernatant tracheal wash obtained from the control swine contained 4 x 10^2 B. bronchiseptica colony-forming units per ml. The serum of the control swine had a titer of 32 while the principal swine had a titer of 8 at necropsy.

On day 116, one principal swine was necropsied and B. bronchiseptica was not recovered from the nasal and tracheal secretions. A serum sample was not obtained. Three swine were necropsied on day 126; a principal, a previously exposed control, and a previously unexposed control. Bordetella bronchiseptica was recovered from the nasal secretions of the previously exposed control swine but not from the principal and unexposed control swine. The principal swine had a serum titer of 64 while serum samples from the 2 control swine were not obtained.

On day 130, the 4 remaining principal swine were inoculated intranasally with virulent B. bronchiseptica (B strain). The 4

principal and 1 previously unexposed control swine of the same age were necropsied on day 137. The nasal secretions did not contain B. bronchiseptica, and the serum titer was negative for the principals and the control swine.

Macroscopic lesions were not observed in the turbinates of any swine. Microscopically, 7 of the principal swine turbinates contained dense focal accumulations of mononuclear cells in the lamina propria while the epithelium appeared normal. The eighth section was lost. Two of the exposed control swine turbinates exhibited epithelial hyperplasia, some cilia loss, microabscesses in the epithelium, and diffuse distribution of mononuclear cells in the lamina propria. Necropsies were not performed on the other 2 exposed control swine. The 2 unexposed control swine turbinates appeared normal microscopically.

Experiment VIII Four 3-week-old pigs were inoculated intranasally on days 1, 5, and 7 with low-virulence B. bronchiseptica (D-1 strain). Nasal secretions were collected on day 17; 1 of 3 pigs was positive for B. bronchiseptica while 1 sample was not interpretable due to bacterial overgrowth. The serum titers were negative for 2 swine and were 4 and 32 for the other swine. All nasal secretions were negative for the organism on day 43. On day 53, the serum titers were negative for 2 swine and were 4 and 8 for the other swine. On days 53, 55, and 57, the 4 principal swine and 2 previously unexposed control swine of the same age were inoculated intranasally with virulent

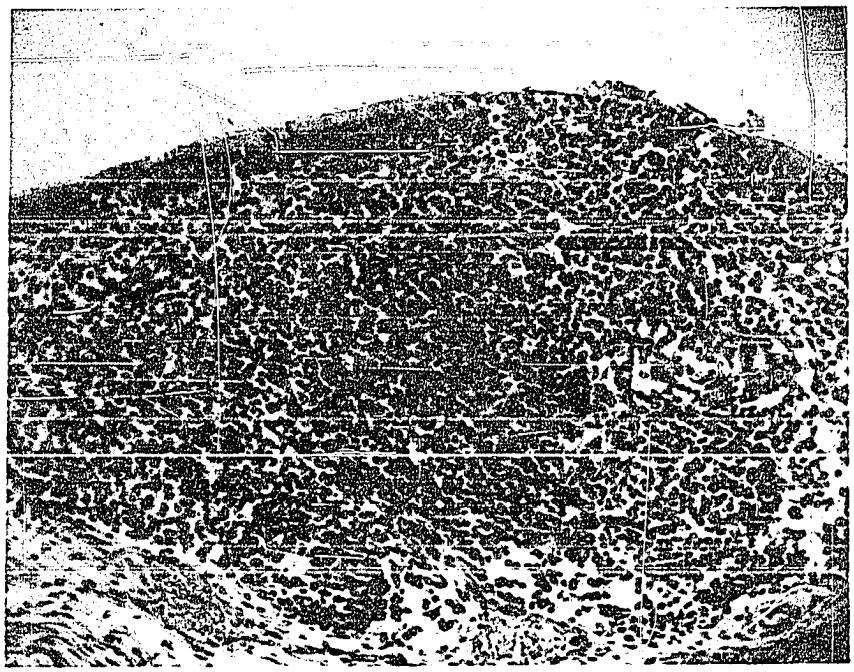
B. bronchiseptica (B strain). Nasal secretions were collected on day 61; the 4 principal swine were negative, and 1 of the 2 control swine was positive for the organism.

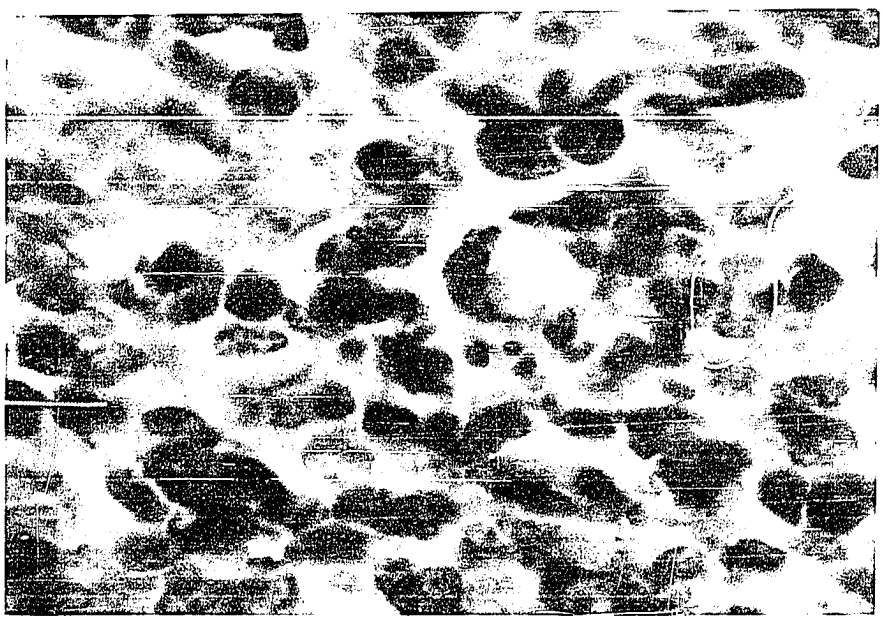
On day 67, the serum titers of 3 principal pigs were 4 (1 sample was not obtained). The 2 control swine serum titers at this time were negative and 32. On days 67, 70, 80, and 112, the 4 principal swine and the same control swine were inoculated intranasally with virulent B. bronchiseptica (B strain). Necropsies were performed on day 130; serum titers for the principal were negative, 4, 4, and 16. Serum titers for the control swine were negative and 16. The nasal secretions were negative for B. bronchiseptica for both principal and control pigs at necropsy.

Macroscopic lesions were not observed. Microscopically, the changes in the turbinates of the principal swine varied from a mild epithelial hyperplasia with a diffuse increase of mononuclear cells in the lamina propria to normal epithelium and focal accumulations of mononuclear cells (Figure 2). The turbinates of the infected controls contained focal accumulations of mononuclear cells in the lamina propria with normal appearing epithelium. All focal accumulations of mononuclear cells contained many pyroninophilic cells (Figure 3). Microscopic lesions were not observed in the tracheas of either principal or control swine.

Figure 2. A focal accumulation of mononuclear cells in the lamina propria of a turbinate from a pig cleared of Bordetella bronchiseptica low-virulence D-1 strain infection and subsequently inoculated intranasally with virulent B strain. This pig was resistant to virulent strain challenge and had been inoculated intranasally with the organism 73 days before necropsy (Experiment VIII). Hematoxylin and eosin stain. 159x

Figure 3. The pyroninophilic nature of a focal accumulation of mononuclear cells from Figure 2 is demonstrated by high magnification (Experiment VIII). Methyl green-pyronine stain. 1240x





A summary of the incidence of B. bronchiseptica recovered from the nasal secretions of principal and control swine from Experiments I, II, III, IV, V, and VIII is presented in Table 9.

Experiment IX Four 2-week-old pigs were inoculated intranasally on days 1 and 3 with low-virulence B. bronchiseptica (D-1 strain). One pig died on day 9 due to starvation caused by early weaning. Nasal secretions collected on day 9, from the 3 remaining swine, were positive for the organism. On days 9, 11, and 14, the 3 principal and 3 unexposed control swine of the same age were inoculated with virulent B. bronchiseptica (strain IVDL 374). Nasal secretions were collected on days 18, 32, 41, 47, 55, and 65; the 3 principal and 3 control pigs were positive for B. bronchiseptica.

Biochemically, cultures of D-1 strain were nitrate negative, and strain IVDL 374 were nitrate positive when inoculated into the experimental swine on days 9, 11, and 14. During the course of the experiment, representative colonies of B. bronchiseptica were picked from the plates previously inoculated with nasal secretions and nitrate reactions determined. The number of colonies picked varied from 1 to 10 per plate.

On day 18, only nitrate positive B. bronchiseptica were recovered from the control swine while both nitrate positive and negative organisms were isolated from the principal swine. On days 32, 41, and 47, the principal swine harbored both nitrate positive and negative B. bronchiseptica; nitrate

Table 9. Summary of the recovery of Bordetella bronchiseptica from the nasal secretions of principal and control swine from Experiments I, II, III, IV, V, and VIII

Treatment	Number of swine	Number of swine positive for B. bronchiseptica between 7 to 11 days after intranasal inoculation with virulent B strain	Chi Square value
Principal swine cleared of B strain infection by sulfonamide therapy	14	0	9.454**
Controls	7	5	
Principal swine cleared of D-1 strain infection by natural elimination	26	0	24.374**
Controls	14	11	

**Significant at $p \leq 0.01$ level.

determinations were not made on the control swine isolates on those days. On days 55 and 65, the principal swine isolates were all nitrate positive; however, the control swine contained some nitrate negative isolates on day 65.

All pigs were necropsied on day 78. The bacteriologic recovery and macroscopic lesions observed at necropsy are presented in Table 10. Microscopically, the turbinates of both control and principal swine had epithelial hyperplasia, cilia loss, and microabscesses in the epithelium. The turbinate osseous core of 2 control swine and 1 principal swine was hypoplastic, and replacement fibrosis was present. The lung of 1 control pig had thickened alveolar walls and peribronchiole fibrosis. The tracheas of both control and principal swine appeared normal.

Experiment X Four 8-week-old pigs were inoculated intranasally on day 1 with 1 ml per pig of a 4 percent acetic acid solution and a broth culture containing a 21st passage of an atypical colony form of low-virulence B. bronchiseptica (D-1 strain). This atypical form grew on MacConkey's agar as a small colony with a large central elevation which had been selected during 21 passages in artificial media. Nasal secretions were collected on day 13; 3 of the 4 swine were positive for the organism. Nasal secretions were collected on day 38; all swine were negative for B. bronchiseptica. On days 38, 40, and 48, the 4 principal swine and 2 unexposed control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain). Nasal secretions were collected on day 48, 2 of the 4 principal and the 2 control swine were positive for the organism. The swine were necropsied on day 53.

Table 10. Incidence of Bordetella bronchiseptica and macroscopic lesions in respiratory tract of swine inoculated with low-virulence D-1 strain and challenged 8 days later with virulent IVDL 374 strain (Experiment IX)

Pig number	Group[a]	Bordetella bronchiseptica			Macroscopic lesions	
		Turbinates	Trachea	Lungs	Turbinate hypoplasia	Pneumonia
61	control	+	-	-	+ (mild)	+
72	control	-	-	-	-	-
65	control	-	-	-	+ (severe)	-
21	principal	-	-	-	+ (mild)	-
73G	principal	-	-	-	-	-
73B	principal	-	-	-	-	-

[a]The principal swine were inoculated with D-1 strain B. bronchiseptica on days 1 and 3. Both principal and control swine were inoculated with IVDL 374 strain of B. bronchiseptica on days 9, 11, and 14.

The nasal secretions of 3 of the 4 principal and the 2 control swine were positive for the organism. Macroscopically, the turbinates appeared normal. Microscopically, both the controls and principal swine turbinates exhibited epithelial hyperplasia with cilia loss. The turbinate osseous core of 1 principal pig was hypoplastic.

Subcutaneous injection of swine with either sonicly disrupted Bordetella bronchiseptica (D-1 strain) vaccine or pertussis-vaccine to induce respiratory resistance

Experiment XI Four 4-week-old pigs were injected subcutaneously with sonicate-vaccine on days 1 and 6. Two of the vaccinated pigs and 2 unvaccinated controls of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 12, 15, and 17. On day 28, nasal secretions from the principal swine were negative and 1 of the control swine was positive for B. bronchiseptica while 1 control swine sample was not interpretable due to bacterial overgrowth. Nasal secretions were collected on days 42 and 79; the vaccinated swine were negative, and the 2 control swine were positive for the organism.

On days 59, 61, and 63, the other 2 vaccinated swine and 2 unvaccinated, unexposed control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain). Nasal secretions were collected on day 70; the 2 vaccinated and the 2 control swine were positive for the organism. Nasal secretions were collected on day 79; the vaccinated

swine were negative, and 1 of the 2 control swine were positive for B. bronchiseptica. Nasal secretions were collected on day 86; 1 of the vaccinated swine and 1 of the control swine were positive for the organism.

Experiment XII Six 4-week-old pigs were injected subcutaneously with sonicate-vaccine on days 1 and 7. One pig died on day 13 of unknown causes. On days 14, 17, and 19, 2 vaccinated swine and 2 unvaccinated control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain). Nasal secretions were collected on days 21, 26, and 41; the 2 vaccinated and the 2 control swine were positive for the organism.

On days 42, 44, and 46, the 3 remaining vaccinated swine and 2 unvaccinated, unexposed swine of the same age were inoculated intranasally with B. bronchiseptica (B strain). Nasal secretions were collected on day 53; 2 of the 3 vaccinated swine and the 2 control swine were positive for the organism. Nasal secretions were collected on day 67; the 3 principal swine were negative while the 2 control swine were positive for B. bronchiseptica.

The swine which were inoculated on days 42, 44, and 46, were necropsied on day 79. Nasal and tracheal secretions from the 3 vaccinated swine were negative for the organism. Nasal secretions from the 2 control swine and tracheal secretions from 1 of the control swine were positive for B. bronchiseptica.

Macroscopic lesions were not observed in the swine. Microscopically, the turbinates of 1 control and 2 vaccinated swine had epithelial hyperplasia with loss of cilia. The trachea of 1 control swine had epithelial hyperplasia with loss of cilia.

Experiment XIII Thirty-six pigs were divided equally into 3 groups based on age as follows: group 1, 2-week-old; group 2, 4-week-old; and group 3, 8-week-old. On day 1 and day 7 or 8, 4 swine in each group were injected subcutaneously with sonicate-vaccine and 4 swine in each group were injected subcutaneously with pertussis-vaccine. The 4 remaining swine in each group served as unvaccinated controls. All swine were inoculated intranasally with virulent B. bronchiseptica (B strain) every other day for 3 days (total of 3 doses). Intranasal inoculations were initiated on day 36 for group 1, day 35 for group 2, and day 38 for group 3. Nasal secretions and blood samples were collected at 2 to 4 week intervals. The animals were necropsied between days 96 and 105.

The incidence of B. bronchiseptica in the nasal secretions of swine during the experiment is presented in Table 11. The average serum agglutinin response against B. bronchiseptica for the unvaccinated, infected control swine and the sonicate-vaccine injected swine is presented in Figure 4. The average serum agglutinin response against B. pertussis for the unvaccinated, infected control swine and the pertussis-vaccine injected swine is presented in Figure 5.

Table 11. Incidence of Bordetella bronchiseptica in the nasal secretions of swine injected with either sonicate- or pertussis-vaccine and inoculated intranasally with virulent B strain (Experiment XIII)

Treatment	Age in weeks at time of first injection of vaccine[a]	Days after first intranasal inoculation of virulent B. bronchiseptica (B strain)				
		0	13-15	25	35-40	61-69
Unvaccinated	2	0/4[b]	4/4	4/4	4/4	3/4
	4	0/4	3/3[c]	N.D.	3/3	2/3
	8	0/4	N.D.	3/3[c]	3/3	3/3
		0/12	7/7	7/7	10/10	8/10
Sonicate-vaccine	2	0/4	4/4	4/4	2/4	0/4
	4	0/4	3/3[d]	N.D.	2/4	0/2[c]
	8	0/4	N.D.	3/4	3/4	1/4
		0/12	7/7	7/8	7/12	1/10
Pertussis-vaccine	2	0/4	3/4	4/4	2/4	1/4
	4	0/4	2/4	N.D.	2/4	1/4
	8	0/4	N.D.	4/4	4/4	1/4
		0/12	5/8	8/8	8/12	3/12

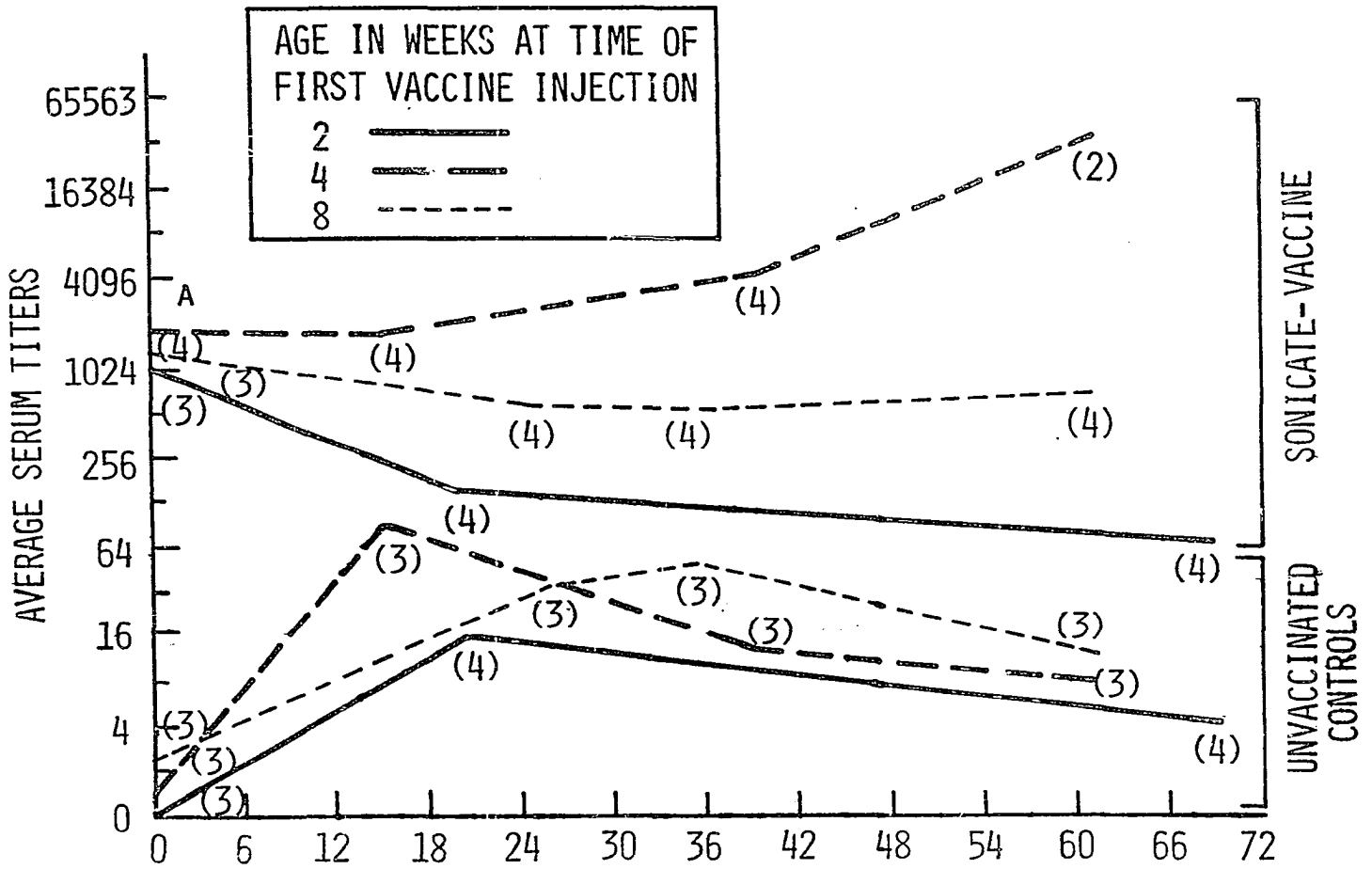
[a]The principal swine were injected with vaccine on days 1 and 7 or 8. All swine were inoculated intranasally with virulent B. bronchiseptica (B strain) between days 35 to 42.

[b]Denominator designates number of swine sampled; numerator designates number of swine positive for B. bronchiseptica.

[c]Sample number decreased due to death of swine during experiment.

[d]One sample not interpretable due to bacterial overgrowth.

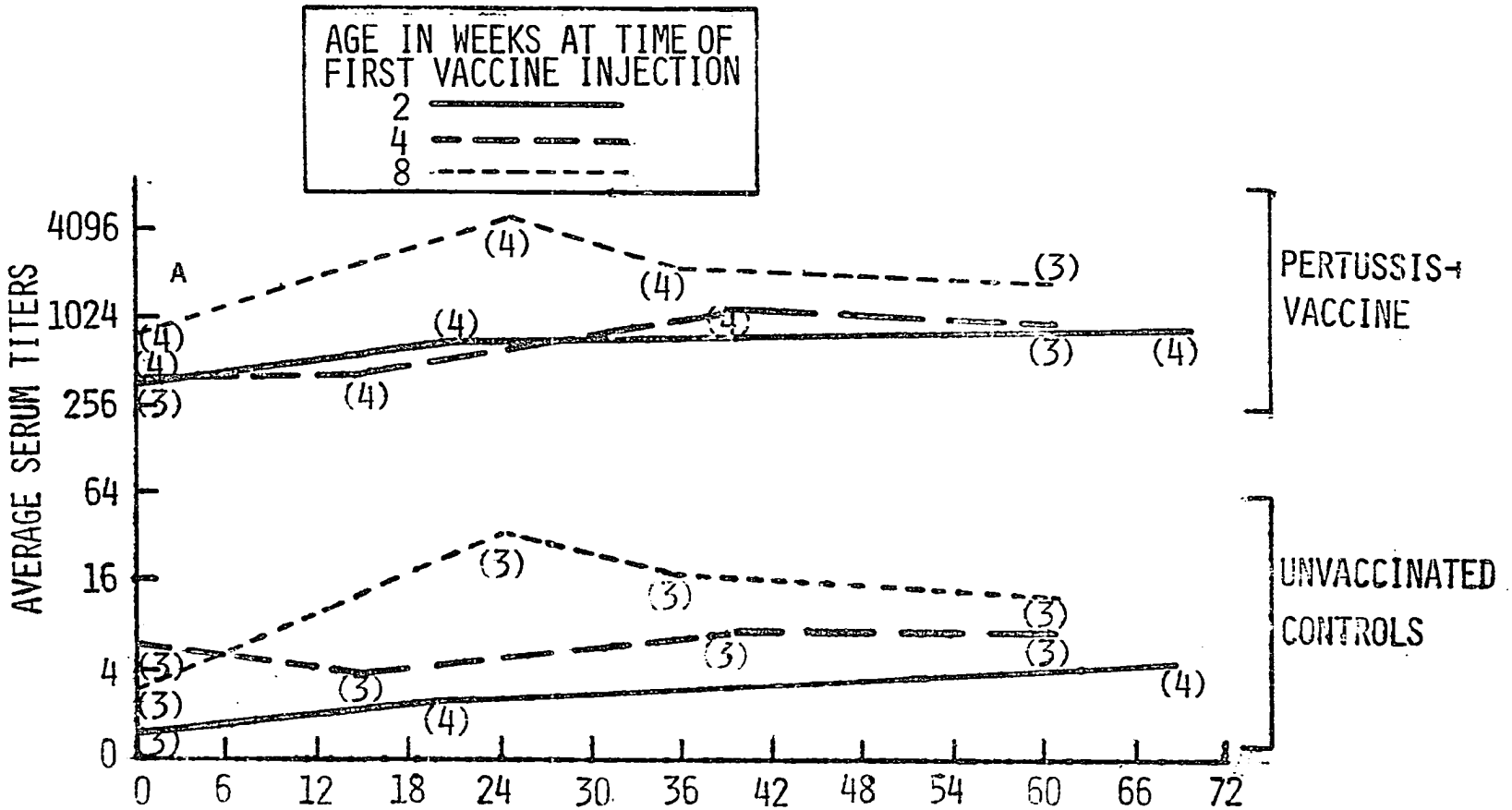
Figure 4. The average serum agglutinin response (PAST) detected against Bordetella bronchiseptica antigen in unvaccinated and sonicate-vaccine injected swine following challenge with virulent B strain (Experiment XIII)



DAYS AFTER FIRST INTRANASAL INOCULATION OF VIRULENT B. BRONCHISEPTICA (B STRAIN).

[A] PARENTHESIS DESIGNATES NUMBER OF SAMPLES

Figure 5. The average serum agglutinin response (PAST) detected against Bordetella pertussis antigen in unvaccinated and pertussis-vaccine injected swine following challenge with virulent B strain (Experiment XIII)



DAYS AFTER FIRST INTRANASAL INOCULATION OF VIRULENT B. BRONCHISEPTICA (B STRAIN).

[A]PARENTHESIS DESIGNATES NUMBER OF SAMPLES.

Macroscopically, turbinate hypoplasia was not present in any of the swine. Microscopically, the turbinates of 5 unvaccinated, infected control swine had epithelial hyperplasia with loss of cilia. The turbinates of both unvaccinated and vaccinated swine had focal accumulations of mononuclear cells in the submucosa. Most focal accumulations of mononuclear cells contained many pyroninophilic cells. Occasionally, a turbinate from vaccinated swine had massive accumulations of mononuclear cells resembling a lymph nodule (Figures 6 and 7).

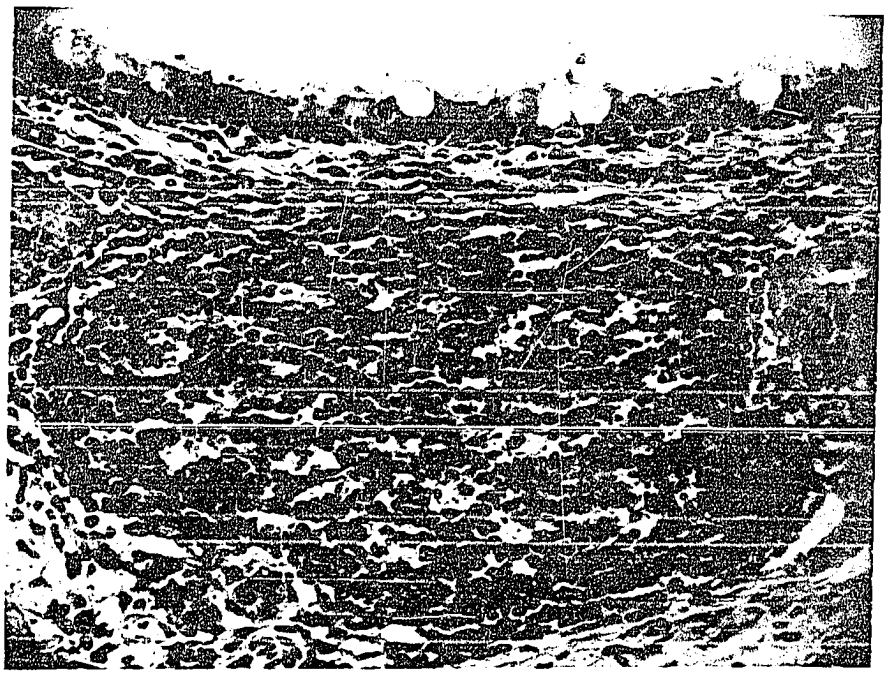
Experiment XIV On days 1 and 6, eight 2-week-old pigs were injected subcutaneously with sonicate-vaccine and four 2-week-old pigs were injected subcutaneously with pertussis-vaccine. Four of the sonicate-vaccine injected swine and 2 unvaccinated control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 44, 46, and 50. The antibody titers for the control swine were 2 and 8 while the titers of the 4 sonicate-vaccine injected swine ranged from 1024 to 4096 at this time.

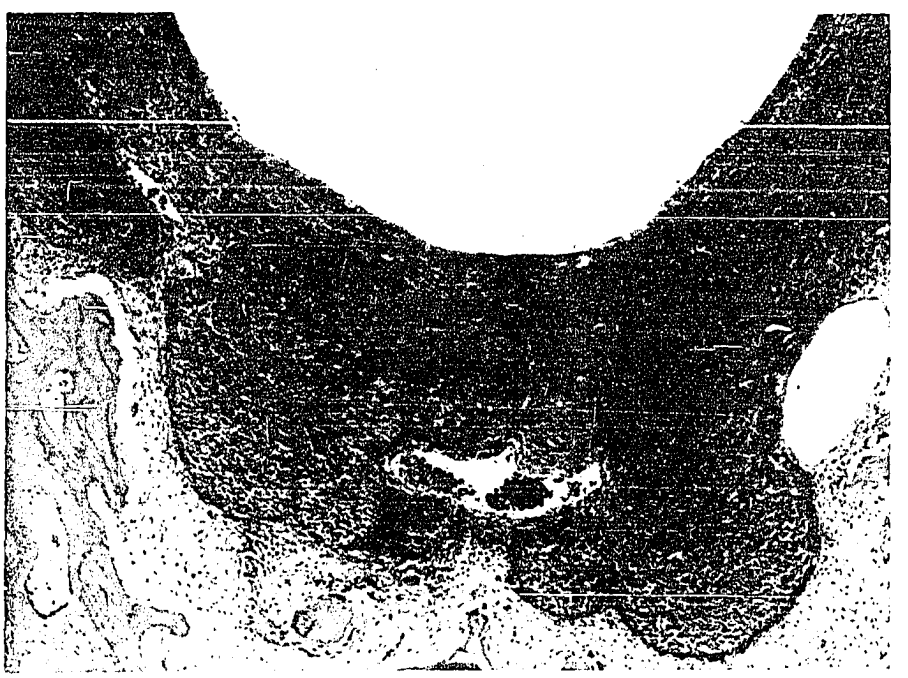
Nasal secretions were collected on day 63; 3 of the 4 sonicate-vaccine injected pigs and 1 of the 2 unvaccinated control swine were positive for the organism. One sample was not interpretable due to bacterial overgrowth. On day 65, one unvaccinated control pig died of unknown causes.

Nasal secretions were collected on day 86; 2 of the 4 sonicate-vaccine injected swine and the 1 unvaccinated control

Figure 6. A focal accumulation of mononuclear cells present in the lamina propria of a turbinate from a pig injected with sonicate-vaccine and subsequently inoculated intranasally with virulent Bordetella bronchiseptica (B strain) (Experiment XIII). Hematoxylin and eosin stain. 244x

Figure 7. An accumulation of mononuclear cells resembling a lymph node present in the lamina propria of a turbinate from a pig injected with pertussis-vaccine and subsequently inoculated intra-nasally with virulent Bordetella bronchiseptica (B strain) (Experiment XIII). Hematoxylin and eosin stain. 124x





pig were positive for the organism. The antibody titer for the control pig was 32 while the titers of the 4 sonicate-vaccine injected swine ranged from 256 to 512 at this time. The swine which were inoculated intranasally on days 44, 46, and 50 were necropsied on day 107. The nasal secretions of the 4 sonicate-vaccine injected swine were negative while the nasal secretions of the 1 unvaccinated control swine were positive for B. bronchiseptica. The antibody titer for the control pig was 16 while the titers of the 4 sonicate-vaccine injected swine ranged from 128 to 512 at the time of necropsy.

The 8 unexposed vaccinated swine (sonicate- and pertussis-vaccine injected) and 4 unvaccinated control swine of the same age were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 116, 118, and 120. The incidence of B. bronchiseptica in the nasal secretions of swine during the experiment is presented in Table 12. The average serum agglutinin response against B. bronchiseptica for the unvaccinated, sonicate-vaccine injected, and pertussis-vaccine injected swine is presented in Figure 8.

A summary and statistical analysis of the data from Experiments XIII and XIV are presented in Table 13.

Experiment XV Six 4-week-old pigs were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 1, 3, and 5. Pertussis-vaccine was injected subcutaneously into 4 of the previously exposed swine on days 7 and 11. On

Table 12. Incidence of Bordetella bronchiseptica in the nasal secretions of swine injected with either sonicate- or pertussis-vaccine before intranasal inoculation with virulent B strain (Experiment XIV)

Treatment	Days after the first intranasal inoculation of virulent B. bronchiseptica (B strain)[a]				
	0	17	40	47	60
Unvaccinated	0/4[b]	4/4	4/4	4/4	2/4
Sonicate-vaccine	0/4	2/4	0/4	0/4	0/4
Pertussis-vaccine	0/4	1/4	1/4	0/3[c]	0/4

[a]The principal swine were injected with vaccine on days 1 and 6. All swine were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 116, 118, and 120.

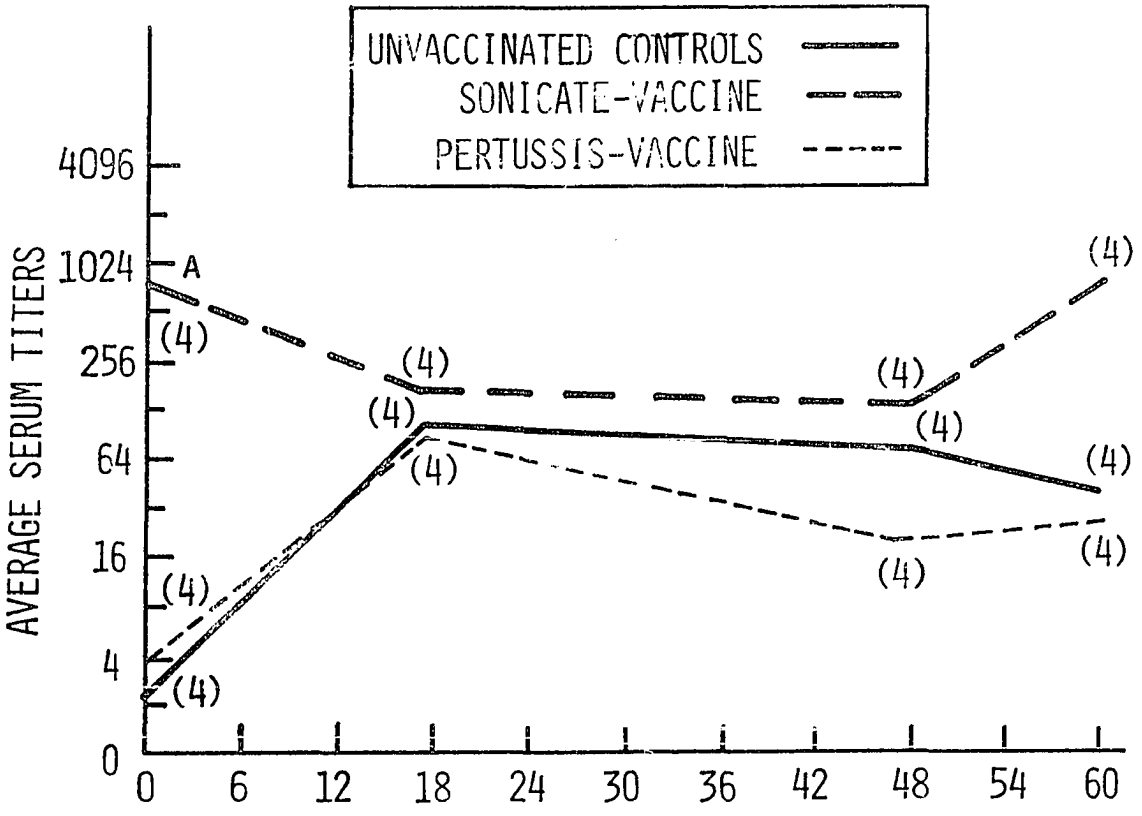
[b]Denominator designates number of swine sampled; numerator designates number of swine positive for B. bronchiseptica.

[c]One sample not interpretable due to bacterial overgrowth.

day 11, nasal secretions of 3 of 3 vaccinated swine and 1 of 1 unvaccinated control swine were positive for the organism. Two samples were not interpretable due to bacterial overgrowth. All swine were positive for the organism on days 20, 28 and 41.

At necropsy on day 59, 3 of 3 vaccinated swine and 2 of 2 unvaccinated control swine were positive for the organism. One sample was not interpretable due to bacterial overgrowth.

Figure 8. The average serum agglutinin response (PAST) detected against Bordetella bronchiseptica antigen in the unvaccinated, sonicate- and pertussis-vaccine injected swine following challenge with virulent B strain (Experiment XIV)



DAYS AFTER FIRST INTRANASAL INOCULATION OF VIRULENT B. BRONCHISEPTICA (B STRAIN).

[A] PARENTHESIS DESIGNATES NUMBER OF SAMPLES.

Table 13. Summary of the incidence of Bordetella bronchiseptica recovered from the nasal secretions of principal and control swine from Experiments XIII and XIV

Treatment	Number of swine	Number of swine positive for B. bronchiseptica between 60-to-69 days after intranasal inoculation of virulent B strain	Chi Square value
Unvaccinated controls	14	10	
Sonicate-vaccine	14	1	9.58**
Pertussis-vaccine	16	3	5.02*

*Significant at $p \leq 0.05$ level.

**Significant at $p \leq 0.01$ level.

Macroscopically, the turbinates of 3 vaccinated swine appeared normal. One vaccinated swine and 1 unvaccinated control swine had mild turbinate hypoplasia while 1 unvaccinated control swine had severe turbinate hypoplasia.

Injection of swine with formalized whole cell Bordetella bronchiseptica vaccines to induce respiratory resistance

Experiment XVI Four 8-week-old pigs were injected intramuscularly with B. bronchiseptica whole cell-vaccine (B strain) on days 1, 5, 10, 15, and 20. On days 28, 30, and 32, the 4 vaccinated swine and 4 unvaccinated control swine of the same age were inoculated intranasally with B. bronchiseptica (B strain). The serum antibody response as determined by rapid plate agglutination (B strain) was 128, 128, 256, and 256 for the vaccinated swine on day 28.

Nasal secretions were collected on day 42. Three of 4 vaccinated swine and 4 of 4 unvaccinated control swine were positive for the organism. Serum antibody response as determined by rapid plate agglutination was 16, 16, 16, and 32 for the vaccinated swine and negative for all 4 unvaccinated swine.

Nasal secretions were collected on day 57; 3 of 4 vaccinated swine and 3 of 4 unvaccinated control swine were positive for B. bronchiseptica. Serum antibody response as determined by rapid plate agglutination was 4, 8, 8, and 16 for the vaccinated swine and negative for all 4 unvaccinated swine at this time.

Experiment XVII Four 5-week-old pigs were injected subcutaneously with B. bronchiseptica whole cell-vaccine (D-1 strain) on days 1 and 8. On days 40, 42, and 44, the 4 vaccinated swine and 2 unvaccinated control swine of the same age were inoculated intranasally with B. bronchiseptica (B strain).

The incidence of B. bronchiseptica in the nasal secretions of swine during the experiment is presented in Table 14. The average serum agglutinin response against B. bronchiseptica in the unvaccinated swine and the whole cell-vaccine injected swine following exposure to virulent B strain B. bronchiseptica is presented in Figure 9.

Subcutaneous injection of pregnant sows with sonicly disrupted Bordetella bronchiseptica (D-1 strain) vaccine to induce respiratory resistance in newborn pigs

This series of observations is based on 4 litters of pigs. Two of these were farrowed by sows that had been vaccinated and 2 were from unvaccinated sows.

Experiment XVIII The first unvaccinated sow farrowed 11 pigs on day 1. On days 3, 5, and 7, the 7 remaining pigs were inoculated intranasally with virulent B. bronchiseptica (B strain). The serum titer of the dam was 8 while the titers of the pigs varied between 2 and 4 on day 3.

Nasal secretions were collected on days 14 and 29. On both occasions 4 pigs were positive for B. bronchiseptica while 3 samples were not interpretable due to bacterial overgrowth. The antibody titers of the pigs on day 14 were negative or 2 while on day 29 the titers ranged from negative to 8.

The pigs were necropsied on day 54 and the serum titers of the pigs were negative or 2. The incidence of B. bronchiseptica in the respiratory secretions and the macroscopic lesions in the pigs at necropsy are presented in Table 15.

Table 14. Incidence of Bordetella bronchiseptica in the nasal secretions of swine injected with whole cell-vaccine (D-1 strain) before intranasal inoculation with virulent B strain (Experiment XVII)

Treatment	Days after the first intranasal inoculation of virulent B. bronchiseptica (B strain)[a]				
	0	12	20	38	60
Unvaccinated	0/2[b]	2/2	2/2	2/2	2/2
Vaccinated	0/4	1/4	2/4	2/4	0/4

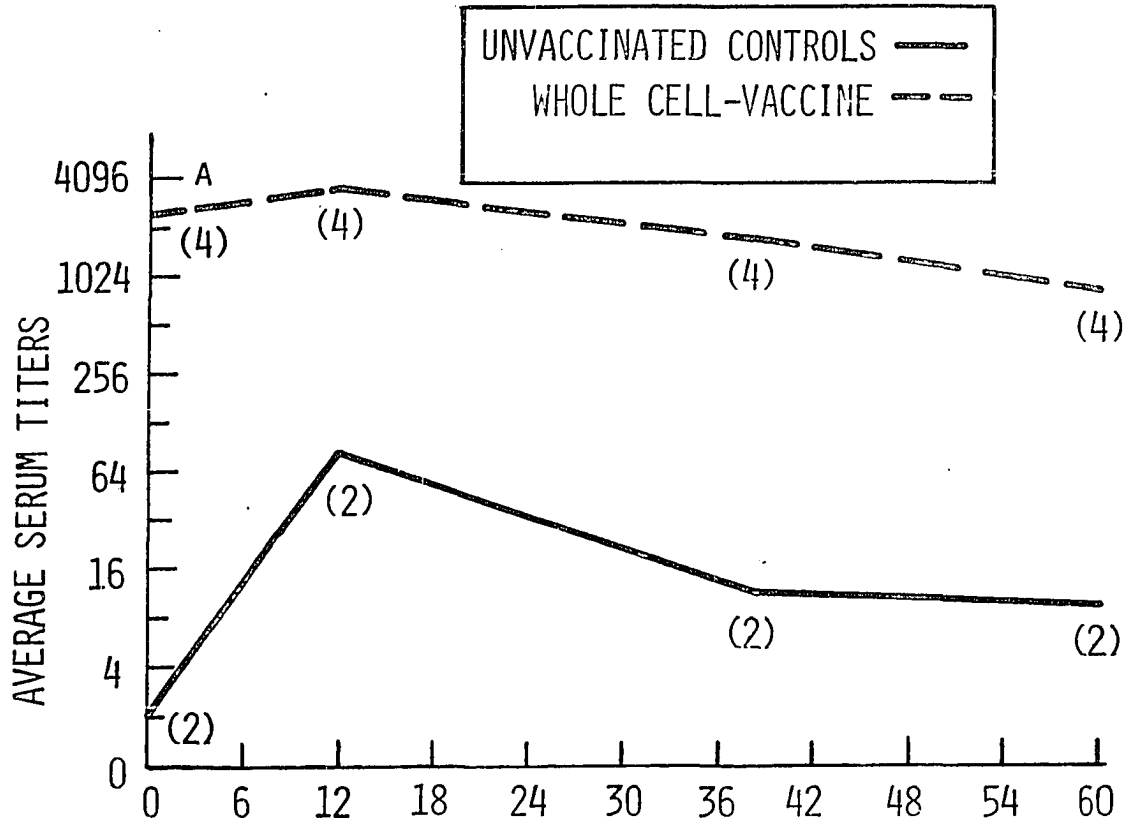
[a]The principal swine were injected with vaccine on days 1 and 8. All swine were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 40, 42, and 44.

[b]Denominator designates number of swine sampled; numerator designates number of swine positive for B. bronchiseptica.

Experiment XIX The second unvaccinated sow farrowed 6 pigs on day 1. On days 3, 6, and 8, the 5 remaining pigs were inoculated intranasally with virulent B. bronchiseptica (B strain). The serum titer of the dam was 4 while the titers of the pigs varied between 8 and 32.

Nasal secretions were collected on day 33, 2 pigs were positive for B. bronchiseptica while 3 samples were not interpretable due to bacterial overgrowth. The serum titers in the pigs at this time were negative. Nasal secretions were

Figure 9. The average serum agglutinin response (PAST) detected against Bordetella bronchiseptica antigen in unvaccinated and whole cell-vaccine (D-1 strain) injected swine following challenge with virulent B strain (Experiment XVII)



DAYS AFTER FIRST INTRANASAL INOCULATION OF VIRULENT B. BRONCHISEPTICA (B STRAIN).

[A] PARENTHESIS DESIGNATES NUMBER OF SAMPLES.

Table 15. The incidence of Bordetella bronchiseptica and macroscopic lesions in the respiratory tract of swine inoculated with virulent B strain after nursing unvaccinated sows (Experiments XVIII and XIX)

Experiment number	Pig number	B. bronchiseptica recovered in respiratory secretions on days 54-71			Macroscopic lesions	
		Turbinates	Trachea	Lung	Turbinate hypoplasia	Pneumonia
XVIII	11G	Overgrown[a]	+	-	+ (severe)	+
	12G	+	+	-	+ (mild)	-
	13G	+	-	+	+ (moderate)	+
	14G	+	+	-	+ (severe)	-
	15G	+	-	-	+ (moderate)	+
	16G	+	+	+	+ (severe)	+
	17G	+	+	+	+ (moderate)	+
XIX	40G	-	-	-	-	-
	40B	+	-	-	+ (mild)	-
	41G	+	-	-	-	-
	41B	-	-	-	+ (mild)	-
	42G	-	-	-	-	-
Totals		8/11[b]	5/12	3/12	9/12	5/12

[a]Sample not interpretable due to bacterial overgrowth.

[b]Denominator designates number of swine sampled; numerator designates number of swine positive.

collected on day 46; 3 pigs were positive for B. bronchiseptica while 2 samples were not interpretable due to bacterial overgrowth.

The pigs were necropsied on day 71. The serum titers in the pigs were 2. The incidence of B. bronchiseptica in the respiratory secretions and the macroscopic lesions in the pigs at necropsy are presented in Table 15.

Experiment XX One pregnant sow was injected subcutaneously with sonicate vaccine 34 and 17 days prior to farrowing. This vaccine was from the same preparation used in Experiment XIV. The serum titer for the sow before vaccination was 16 while 26 days after the first vaccination the titer was 1024. Eleven pigs were farrowed on day 1 which was the 34th day after the first vaccination. On days 2, 6, and 8 the 10 remaining pigs were inoculated intranasally with virulent B. bronchiseptica (B strain). The serum titer of the dam was 2048 while the titers of the pigs ranged from 512 to 4096 at the time of the first inoculation.

Nasal secretions were collected from the pigs on days 14 and 22, on both occasions all 10 pigs were positive for B. bronchiseptica. Nasal secretions were collected on day 70, 8 of the 10 pigs were positive for the organism. The serum titers of the pigs ranged from 16 to 128 at this time.

The pigs were necropsied on day 99. The serum titers of the pigs ranged from 4 to 128. The incidence of B. bronchiseptica in the respiratory secretions and the macroscopic lesions

in the pigs at necropsy are presented in Table 16.

Experiment XXI The second pregnant sow was injected subcutaneously with sonicate-vaccine 42 days and again 25 days prior to farrowing. This vaccine was from the same preparation used in Experiment XIV. The serum titer for the sow prior to vaccination was 2 while 26 days after the first vaccination the titer was 1024. Five pigs were farrowed on day 1. On days 2, 6, and 8, the 4 remaining pigs were inoculated intranasally with virulent B. bronchiseptica (B strain). The serum titer in the dam was 4096 while the titers of the pigs were all 4096 when the pigs were 2 days of age.

Nasal secretions were collected from the pigs on days 14 and 28. All 4 were positive for the organism. The serum titers of the pigs ranged from 256 to 1024 on day 28.

The pigs were necropsied on day 57 at which time the serum titers of the pigs ranged from 32 to 256. The incidence of B. bronchiseptica in the respiratory secretions and the macroscopic lesions in the pigs at necropsy are presented in Table 16.

Respiratory resistance to Bordetella bronchiseptica associated with age of swine at the time of first intranasal exposure

Experiment XXII Four 3-week-old pigs (Group 1) and four 24-week-old pigs (Group 2) were inoculated with virulent B. bronchiseptica (B strain) on days 1, 3, and 6. Two principal swine from each age group and 2 previously unexposed control

Table 16. The incidence of Bordetella bronchiseptica and macroscopic lesions in the respiratory tract of swine inoculated with virulent B strain after nursing sonicate-vaccine injected sows (Experiments XX and XXI)

Experiment number	Pig number	B. bronchiseptica recovered in respiratory secretions on days 57-91			Macroscopic lesions	
		Turbinates	Trachea	Lung	Turbinate hypoplasia	Pneumonia
XX	20G	-	-	-	-	-
	20B	+	-	-	+ (moderate)	-
	21G	-	-	-	+ (mild)	-
	21B	+	-	-	+ (mild)	-
	22G	-	-	-	-	-
	22B	-	-	-	+ (mild)	-
	23G	+	+	-	+ (mild)	-
	23B	+	-	-	-	-
	24G	-	-	-	-	-
	24B	+	-	-	-	-
XXI	30G	-	-	-	+ (mild)	-
	30B	-	-	-	+ (mild)	-
	31B	+	-	-	+ (moderate)	-
	32B	+	+	-	+ (moderate)	-
Totals		7/14[a]	2/14	0/14	9/14	0/14

[a]Denominator designates number of swine sampled; numerator designates number of swine positive.

swine of the same age for each group were necropsied on day 45. Macroscopic lesions of turbinate hypoplasia of moderate and severe degrees were observed only in the two principal swine from group 1. Macroscopic lesions were not observed in the respiratory tracts of the other swine. The incidence of B. bronchiseptica in the nasal secretions of the swine during the experiment is presented in Table 17.

The 2 remaining principal swine in group 2 which had cleared the infection by day 33, and 2 previously unexposed swine of the same age were inoculated with virulent B. bronchiseptica (B strain) on days 59, 61, and 63. Nasal secretions were collected on day 67; the principal swine were negative, and the control swine were positive for the organism. These 4 swine were necropsied on day 69 and macroscopic lesions were not observed in the respiratory tracts. The nasal secretions of all 4 swine were positive for B. bronchiseptica at this time.

Serologic Tests

Development of the particulate antigen settling test (PAST)

Initial attempts to utilize a tube agglutination test for the detection of swine antibodies against B. bronchiseptica were disappointing in that the sensitivity of the test system was apparently low. The tube agglutination test procedure was transferred to disposable plastic trays in order to facilitate serum dilution with micro-titration equipment and to increase ease of

Table 17. Incidence of Bordetella bronchiseptica in the nasal secretions of 3-week-old and 24-week-old swine inoculated intranasally with virulent B strain (Experiment XXII)

Age at the time of the first intranasal inoculation[a]	Days after the first intranasal inoculation				
	0	12	24	33	45[b]
3 weeks	0/4[c]	1/2[d]	4/4	4/4	1/1[d]
24 weeks	0/4	3/4	4/4	0/4	0/2

[a]All swine were inoculated intranasally with virulent B. bronchiseptica (B strain) on days 1, 3, and 6.

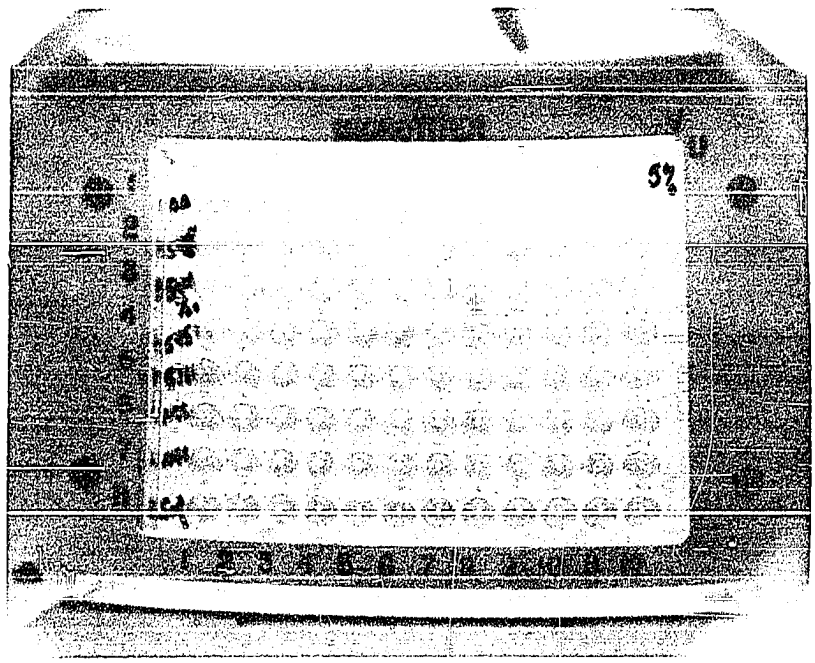
[b]Two swine in each age group were sampled on day 45.

[c]Denominator designates number of swine sampled; numerator designates number of swine positive for B. bronchiseptica.

[d]Some samples not interpretable due to bacterial overgrowth.

microscopic examination of the agglutination reaction. Agglutination of the antigen by antibodies against B. bronchiseptica was observed microscopically after incubation of the test plates at 37° C for 2 hours. Further incubation of the test plates at 4-8° C for 14 to 24 hours allowed for settling of the antigen in the form of either shields (antibody present) or buttons (antibody absent) in the wells of the plate (Figure 10). Preliminary studies with this system revealed a considerable increase in titer for various sera containing antibody against

Figure 10. The endpoints of the particulate antigen settling test were determined by the formation of shields (antibody present) and buttons (antibody absent). The sera in rows 1, 2, 3, 4, and 5 were from experimental pigs which had been exposed to Bordetella bronchiseptica either by injection of a vaccine or by intranasal inoculation of the live organism. The sera in rows 6 and 7 were from unvaccinated, unexposed pigs. Row 8 contained 0.85 percent NaCl solution in place of serum



B. bronchiseptica. Therefore, some of the parameters of the system called the particulate antigen settling test (PAST) were determined.

Diluents containing various concentrations of sodium chloride were compared in the PAST with B. bronchiseptica antigens using pooled serum samples from unexposed control, vaccinated, and infected swine. The titers obtained when a diluent containing either 5.0 percent or 0.85 percent sodium chloride was used are presented in Table 18. A diluent containing 5.0 percent sodium chloride was optimal for the PAST when B. bronchiseptica antigens were used. By contrast, a diluent containing 2.5 percent sodium chloride was optimal for the PAST when Salmonella spp. antigens were used. PAST titers to B. bronchiseptica obtained with sera from unvaccinated, unexposed swine of various ages are presented in Table 19.

Live and formalin inactivated antigens of B. bronchiseptica strains D-1 and B were prepared from agar and broth cultures. PAST titers obtained with these antigens are presented in Table 20. A 1:1000 concentration of formalin was found to be optimal for inactivation of the antigen when incubated at 37° C for 24 hours prior to harvest.

The reproducibility of the PAST was evaluated utilizing a formalin inactivated B. bronchiseptica (D-1 strain) broth culture antigen. Four lots of antigen which were prepared in a similar way on different days are compared in Table 21. The

Table 18. Effect of sodium chloride concentration of the diluent on the particulate antigen settling test[a]

Sample number[b]	Exposure[c] and/or vaccination experience of donor swine	Diluent 0.85% NaCl	Diluent 5.0% NaCl
1	whole cell-vaccine (B strain) - exposed to B strain	4096[d]	512
543	pertussis-vaccine - exposed to B strain	512	256
544	sonicate-vaccine - exposed to B strain	32,768	16,384
545	unvaccinated - exposed to B strain	16	32
530	D-1 strain cleared - exposed to B strain	64	32
572	unexposed control	32	8
422	unexposed control	64	4

[a]Formalin inactivated B. bronchiseptica (D-1 strain) broth culture.

[b]Refer to Table 2 for history of sample source.

[c]Intranasal inoculation with B. bronchiseptica (virulent B strain or low-virulence D-1 strain).

[d]Titers expressed as the reciprocal of the serum dilution.

formalin inactivated broth antigen (D-1 strain) was stable for at least 90 days when stored at 4-8° C.

Swine and rabbit antisera against formalin inactivated broth B. bronchiseptica (D-1 strain) antigen were compared in

Table 19. Titers detected by the particulate antigen settling test in serum from unvaccinated, unexposed swine in relation to the age of the animal[a]

Titer[b]	Age at time of collection					Total
	3 days	2-4 weeks	4-8 weeks	8-16 weeks	over 16 weeks	
<2	-[c]	18	9	4	-	31
2	1	4	18	9	1	33
4	7	3	3	2	1	16
8	2	1	-	-	1	4
16	2	-	1	2	3	8
32	1	-	-	-	-	1
Total	13	26	31	17	6	93

[a]Formalin inactivated B. bronchiseptica (D-1 strain) broth culture.

[b]Titer expressed as the reciprocal of the serum dilution.

[c]Number of swine per age group.

the PAST, tube, plate, and microagglutination tests (MAT) (Table 22). Additional swine sera were tested with formalin inactivated broth B. bronchiseptica (D-1 strain) antigen in both the PAST and plate agglutination tests (Table 23). Of the 21 unvaccinated, infected control swine serum samples recorded in Table 16, 20 pigs contained titers of 8 or more in the PAST as compared to only 2 pigs in the plate agglutination test.

Table 20. Comparison of various antigen preparations of Bordetella bronchiseptica in the particulate antigen settling test

Sample number[a]	Antigen preparation							
	D-1 strain				B strain			
	Live, agar	Live, broth	Inact.,[b] agar	Inact., broth	Live, agar	Live, broth	Inact., agar	Inact., broth
1	1024[c]	512	512	1024	512	2048	512	1024
543	128	512	8	512	512	64	4	512
544	512	16,384	512	16,384	512	32,768	512	8,192
545	--	8	32	16	4	8	32	8
530	8	32	8	32	2	4	2	16
422	--	4	4	4	--	2	--	4
532	--	16	4	2	--	4	2	2

[a]Refer to Table 2 for history of sample source.

[b]Formalin inactivated.

[c]Titer expressed as the reciprocal of the serum dilution.

Table 21. Comparison of the reproducibility of different lots of the inactivated broth antigen of Bordetella bronchiseptica (D-1 strain)

Sample number[a]	Antigen preparation				
	A	B	C	D	E
1	2048[b]	256	256	256	128
	1024	512	256	256	128
		256			
543	512	256	128	128	256
	N.D.	256	128	256	256
		128			
544	16,384	65,536	16,384	8,192	8,192
	16,384	32,768	4,096	8,192	8,192
		32,768			
530	16	16	16	N.D.	N.D.
	32	N.D.	16	N.D.	N.D.
		N.D.			
422	4	8	4	8	4
	2	4	8	4	2
		N.D.			
532	4	2	4	2	2
	8	4	8	2	2
		8			

[a]Refer to Table 2 for history of sample source.

[b]Titer expressed as the reciprocal of the serum dilution.

Results obtained with Salmonella spp. swine and rabbit antisera in the PAST and the tube agglutination tests are presented in Table 24.

PAST titers to B. bronchiseptica obtained with pooled nasal wash samples from swine are presented in Table 25.

Table 22. Comparison of the particulate antigen settling test, tube, plate and microagglutination tests[a] with swine and rabbit anti-Bordetella bronchiseptica serum

Sample number[b]	Species	Type of serologic test				
		Pipette dilution			Microdiluter	
		Tube	Plate	PAST	PAST	MAT
1	Swine	--[c]	4	512	512	2
544	Swine	512	512	2048	8192	512
575	Swine	256	512	1024	2048	32
576	Swine	--	--	16	32	8
685	Swine	--	--	32	64	2
686	Swine	16	16	256	256	8
691	Swine	--	--	64	64	4
693	Swine	--	--	--	2	--
694	Swine	--	--	--	4	--
633	Rabbit	256	256	2048	8192	512
634	Rabbit	--	32	N.D.	2048	--
591	Rabbit	--	--	N.D.	8	--
592	Rabbit	--	--	N.D.	--	--

[a]Formalin inactivated B. bronchiseptica (D-1 strain) broth culture.

[b]Refer to Tables 2 and 3 for history of sample source.

[c]Titer expressed as the reciprocal of the serum dilution.

Table 23. Individual serum antibody response in swine from Experiments XIII and XIV as determined by the particulate antigen settling test and plate agglutination[a]

Sample number	Treatment of sample source	Type of serologic test	
		Plate	PAST
70	Unvaccinated-Unexposed[b]	-[c]	-
71	" "	-	-
74	" "	-	-
167	" "	-	4
168	" "	-	-
171	" "	-	2
231	" "	-	2
233	" "	8	-
239	" "	-	-
241	" "	-	2
621	" "	4	8
626	" "	32	2
631	" "	-	4
632	" "	-	-
82	Unvaccinated-Infected[b]	2	32
85	" "	-	64
86	" "	-	32
87	" "	-	32
204	" "	4	64
209	" "	2	32
258	" "	2	256
259	" "	4	256
261	" "	4	64
648	" "	4	256
649	" "	8	64
650	" "	-	128
651	" "	-	4
661	" "	-	32
662	" "	-	64
664	" "	-	32
666	" "	-	16

[a]Formalin inactivated B. bronchiseptica (D-1 strain) broth culture.

[b]Designates if intranasal inoculation of B. bronchiseptica (B strain) was conducted.

[c]Titer expressed as the reciprocal of the serum dilution.

Table 23 (Continued)

Sample number	Treatment of sample source	Type of serologic test: Plate	Type of serologic test: PAST
687	Unvaccinated-Infected[b]	-	16
688	" "	8	32
689	" "	-	32
690	" "	-	32
623	Sonicate vaccinated-Unexposed[b]	-	256
624	" " "	16	1024
628	" " "	8	256
629	" " "	8	128
642	Sonicate vaccinated-Exposed[b]	8	256
643	" " "	4	128
644	" " "	16	1024
647	" " "	8	128
660	" " "	16	128
667	" " "	8	64
668	" " "	-	16
670	" " "	8	64

Direct hemagglutination inhibition test

Results obtained with the direct hemagglutination test on nasal wash samples obtained from swine are presented in Table 25.

Opsonocytophagic test

The opsonocytophagic test was performed on pooled serum and nasal wash samples obtained from several animal experiments. The results of these determinations are presented in Table 26.

Table 24. Comparison of the particulate antigen settling test, tube, and microagglutination tests with swine and rabbit anti-*Salmonella spp.* serum

Sample number[a]	Species	Antigen	Type of serologic test		
			Tube	PAST	MAT
33	Rabbit	*S. virginia* H	256[b]	256	256
33	Rabbit	*S. virginia* H	256	512	512
NRS[c]	Rabbit	*S. virginia* H	-	-	4
NRS	Rabbit	*S. virginia* H	-	-	4
34	Rabbit	*S. virginia* O	64	256	256
34	Rabbit	*S. virginia* O	32	512	128
NRS	Rabbit	*S. virginia* O	-	-	4
NRS	Rabbit	*S. virginia* O	-	-	8
37	Rabbit	*S. binza* H	512	1024	512
37	Rabbit	*S. binza* H	512	2048	1024
NRS	Rabbit	*S. binza* H	-	8	8
NRS	Rabbit	*S. binza* H	-	4	-
38	Rabbit	*S. binza* O	1024	512	256
38	Rabbit	*S. binza* O	1024	1024	1024
NRS	Rabbit	*S. binza* O	-	8	-
NRS	Rabbit	*S. binza* O	-	-	4
614	Swine	*S. binza* H	512	128	128
614	Swine	*S. binza* H	1024	128	128
573[d]	Swine	*S. binza* H	4	16	8
573	Swine	*S. binza* H	8	8	N.D.
615	Swine	*S. binza* O	256	256	128
615	Swine	*S. binza* O	128	128	N.D.
574[d]	Swine	*S. binza* O	8	4	-
574	Swine	*S. binza* O	8	4	N.D.

[a]Refer to Tables 2 and 3 for history of sample source.
[b]Titer expressed as the reciprocal of the serum dilution.
[c]Normal Rabbit Serum (No pre-injection sample available).
[d]Pre-injection serum sample.

Table 25. Titer of swine nasal wash collections as determined by the particulate antigen settling test[a] and direct hemagglutination inhibition

Sample number[b]	Type of serologic test	
	PAST	HAI
308	1[c]	-
309	1	1
310	-	-
379	-	1
380	2	N.D.
381	32	1
435	1	1
436	-	1
437	1	1
438	1	2
442	8	-
443	-	-
456	-	-
457	2	1
472	8	-
473	2	-
474	-	1
475	2	1
489	-	N.D.
493	-	N.D.
499	-	-
500	-	-
533	-	-
534	4	N.D.
652	-	N.D.
653	-	-

[a]Formalin inactivated B. bronchiseptica (D-1 strain) broth culture.

[b]Refer to Table 4 for history of sample source.

[c]Titer expressed as the reciprocal of the nasal wash dilution.

Table 26. Results of opsonocytophagic test on pooled sera and nasal wash samples

Sample number[a]	Sample type	Averaged opsonocytophagic test values			
		Phagocytic index		Percent phagocytosis	
		B strain	D-1 strain	B strain	D-1 strain
422	Serum	2.63[2][b]	1.73[3]	80.0[2]	92.0[3]
1	Serum	13.95[2]	24.85[2]	100.0[2]	97.0[2]
424a	Serum	4.94[1]	14.00[1]	86.0[1]	100.0[1]
460a	Serum	5.08[1]	18.48[1]	90.0[1]	100.0[1]
466a	Serum	4.96[1]	8.92[1]	96.0[1]	98.0[1]
383a	Serum	4.18[1]	10.80[1]	94.0[1]	100.0[1]
142	Serum	9.10[1]	21.15[2]	86.0[1]	94.0[1]
576	Serum	4.33[2]	11.73[2]	88.0[2]	98.0[2]
533	Nasal wash	2.51[4]	6.63[4]	70.5[4]	85.0[4]
499	Nasal wash	1.90[2]	7.25[2]	59.0[2]	83.0[2]
457	Nasal wash	1.80[1]	10.5[1]	68.0[1]	88.0[1]
500	Nasal wash	1.92[2]	6.96[2]	70.0[2]	87.0[2]
534	Nasal wash	4.00[1]	8.60[1]	68.0[1]	88.0[1]
456	Nasal wash	3.03[2]	4.27[2]	81.0[2]	87.0[2]
442	Nasal wash	2.30[1]	21.40[1]	88.0[1]	100.0[1]

[a]Refer to Tables 2 and 4 for history of sample source.

[b]Superscript designates the number of determinations.

Bactericidal test

Nasal and tracheal wash was collected from a pig at necropsy on day 107 of Experiment VII. This animal had been infected with and had cleared low-virulence B. bronchiseptica (D-1 strain) and was resistant to intranasal inoculation by virulent B strain. The wash material contained hemoglobin. Swine complement was not included in the bactericidal test. The nasal and tracheal wash and a tryptose phosphate broth control were incubated in separate tubes with B. bronchiseptica cells. The nasal wash and tryptose phosphate broth tubes contained 12 colony-forming units per ml while the tracheal wash tube contained 14 colony-forming units per ml.

Nasal wash was obtained from the 4 principal swine in Experiment VIII on day 105 and from previously unexposed swine of the same age. The 4 principal swine had been infected and cleared of low-virulence D-1 strain and were resistant to intranasal inoculation by virulent B strain. The nasal washes did not contain hemoglobin and were held in an ice bath during concentration. The nasal washes were concentrated 2X and sterilized by cellulose acetate membrane filtration. No swine complement was included in the bactericidal test. Results obtained in the bactericidal test with this material are presented in Table 27.

The bactericidal test was performed utilizing guinea pig complement with the nasal wash collected on day 105 of

Table 27. Results of bactericidal test on pooled sera and nasal wash samples

Sample number[a]	Sample type	Complement[b]	Averaged colony-forming units per mililiter
499	Nasal wash	-	70^{1}[c]
500	Nasal wash	-	66^{1}
499	Nasal wash	+	34.00^{3}
500	Nasal wash	+	32.67^{3}
422	Serum	+	36.67^{3}
1	Serum	+	12.67^{3}

[a]Refer to Tables 2 and 4 (methods) for history of sample source.

[b]Indicates if swine complement was added.

[c]Superscript designates the number of determinations.

Experiment VIII. The average of 3 bactericidal tests conducted on different days are presented in Table 27.

Agglutination of Erythrocytes and Epithelial Cells

Direct hemagglutination by B. bronchiseptica was investigated because the organism may attach to the respiratory mucosa by the same mechanism involved in erythrocyte agglutination. It is possible that the ability of the organism to attach to cells may explain certain aspects of both the pathogenesis and resistance to B. bronchiseptica infection.

Bordetella bronchiseptica whole cells hemagglutinated swine, human, sheep, and guinea pig erythrocytes. Bordetella pertussis whole cells hemagglutinated swine and human erythrocytes. Haemophilus spp. hemagglutinated swine erythrocytes while Pasteurella multocida, serotype D, did not hemagglutinate swine erythrocytes.

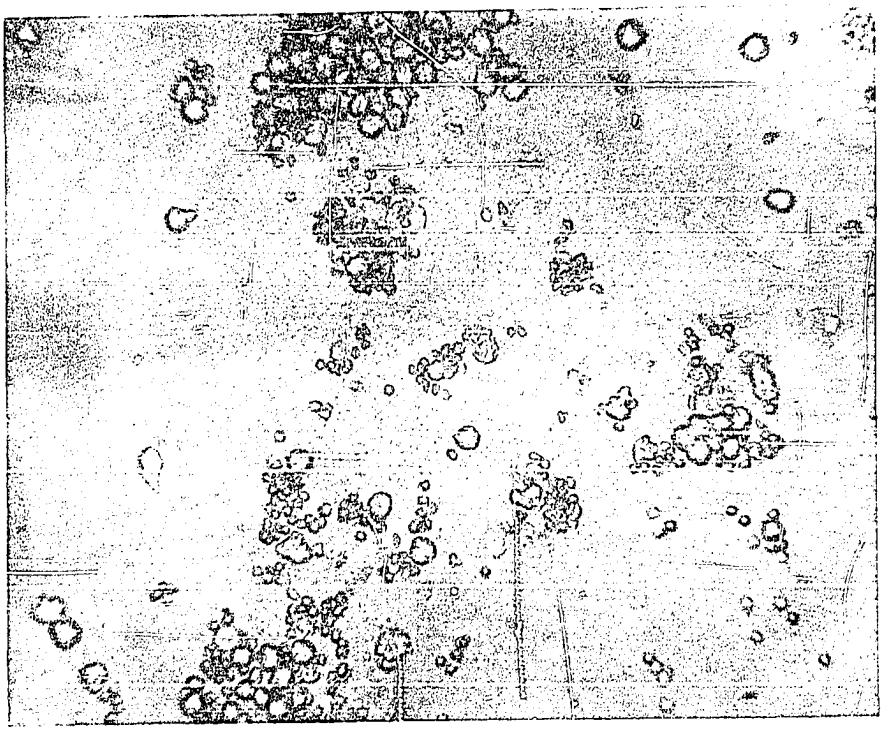
Low-virulence (D-1 strain, 9th passage) and virulent (B strain, 9th passage) B. bronchiseptica whole cells were harvested from horse blood agar cultures. The D-1 strain harvest contained 2.3×10^9 colony-forming units per ml and a hemagglutination titer of 128. The B strain harvest contained 2.2×10^{10} colony-forming units per ml and a hemagglutination titer of 64. The hemagglutination titer was found to decrease in both the D-1 strain and the B strain on continued passage on artificial medium.

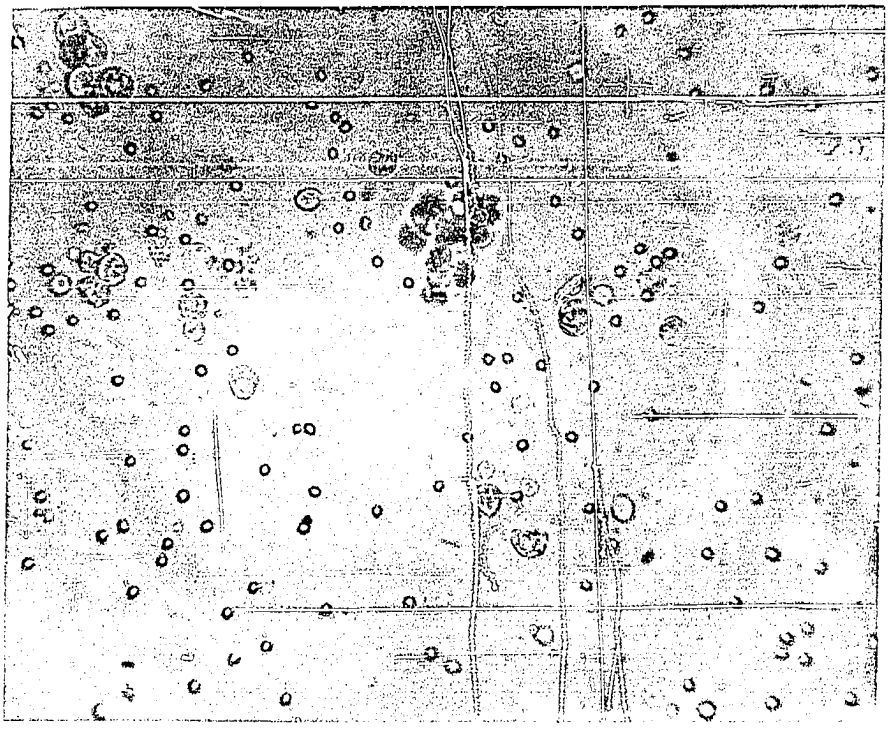
Swine nasal epithelial cells were collected at necropsy from a 2-week-old previously unexposed control pig. Concentrated B. bronchiseptica whole cells were mixed with the epithelial cells and sheep red blood cells; and the mixture was incubated 30 minutes at 37° C.

The swine nasal epithelial cells which were incubated with both B. bronchiseptica whole cells and sheep erythrocytes were surrounded by rosettes of erythrocytes when viewed in wet mount preparations at 100x magnification (Figure 11). When swine nasal epithelial cells were incubated with only sheep erythrocytes, rosette formation did not occur (Figure 12).

Figure 11. An unstained wet mount preparation containing swine nasal cavity epithelial cells surrounded by rosettes of sheep erythrocytes due to the presence of Bordetella bronchiseptica. 430x

Figure 12. An unstained wet mount preparation containing swine nasal cavity epithelial cells and sheep erythrocytes. Bordetella bronchiseptica was not added to the suspension. 430x





Phagocytosis of Bordetella bronchiseptica

The ability of swine leucocytes to phagocytize B. bronchiseptica was determined in order to evaluate certain aspects of both pathogenesis and resistance to infection by the organism.

Bordetella bronchiseptica cells were phagocytized by swine polymorphonuclear cells without hyperimmune or normal sera. The phagocytic index and percent phagocytosis were calculated by averaging the values obtained from 4 determinations conducted on different days. The phagocytic index was 3.10 for virulent B strain and 11.48 for low-virulence D-1 strain. The percent phagocytosis was 82.50 for virulent B strain and 90.25 for low-virulence D-1 strain. The values of the individual determinations are presented in Table 28.

Table 28. Results of phagocytosis of virulent (B strain) and low-virulence (D-1 strain) B. bronchiseptica by swine polymorphonuclear leucocytes

Determination	Phagocytic index		Percent phagocytosis	
	B strain	D-1 strain	B strain	D-1 strain
1	3.40	11.30	96.0	93.0
2	2.00	14.10	66.0	92.0
3	3.18	11.54	86.0	88.0
4	3.82	8.98	82.0	88.0
Average	3.10	11.48**	82.50	90.25 N.S.

**Significant at $p \leq 0.01$ level.

DISCUSSION

Resistance to intranasal infection by B. bronchiseptica was induced in pigs by 2 routes of inoculation. Other workers have reported the use of immunization procedures to reduce the clinical manifestations of pneumonia in swine caused by B. bronchiseptica (Phillips, 1944 and Ray, 1950). However, the first clear demonstration of the induction of resistance in swine to infection by the organism was reported recently and is included in this dissertation (Harris and Switzer, 1969).

Resistance to nasal reinfection by B. bronchiseptica was induced in 14 of 14 pigs by clearing the nasal cavity of a virulent B strain infection by sulfonamide therapy (Table 9). These results suggested that once infected swine had eliminated the organism, refractiveness to reinfection occurred. It has been reported that exposure of young pigs to virulent B. bronchiseptica results in chronic infection (20 weeks) which is not readily eliminated from the respiratory tract (Ross, 1965). However, Ross et al. (1967) demonstrated that D-1 strain B. bronchiseptica would not cause macroscopic lesions in the respiratory tract of young pigs and that the organism was eliminated from the respiratory secretions within 4 to 5 weeks after initial exposure. In the present study, nasal resistance to reinfection was induced in 26 of 26 pigs by infecting them with the low-virulence D-1 strain (Table 9). The pigs were challenged with virulent B strain after clearance of the D-1

strain had occurred. These results contrast with the nasal resistance produced in humans by previous infection and clearance of Staphylococcus aureus infection. Ehrenkranz (1966) demonstrated that once an individual had cleared an infection of the organism, the individual was resistant to homologous strain but not to heterologous strain challenge. Therefore, Ehrenkranz concluded that an immunologic specificity was associated with resistance to reinfection in the nasal cavity. The antigenic relationship of D-1 and B strain have not been determined the 2 strains could be immunologically related with regard to resistance.

Twenty-six,low-virulence D-1 strain cleared,resistant pigs were utilized to characterize various parameters of the resistance to reinfection. Nasal resistance persisted despite repeated intranasal inoculations of virulent B strain (Experiment III) and continued for at least 125 days after initial exposure to the low-virulence D-1 strain (Table 5). Both nasal and tracheal resistance to infection by virulent B strain was induced by a previous nasal infection with the low-virulence D-1 strain (Table 6). No conclusions could be made with regard to lung resistance because the virulent B strain did not infect the lungs of all positive control swine. Andersen (1953) has demonstrated a similar refractiveness to reinfection in mice infected with B. pertussis. Mice inoculated intranasally with sublethal doses of B. pertussis cleared the organism from

their lungs in 59 days. These mice were resistant to reinfection as determined by culturing the lungs 4- to 8-days after challenge with lethal doses of the organism.

Experiment VII was designed to determine the rate of clearance of virulent B strain from resistant pigs and to assess the association of the organism with cells of the swine nasal cavity. Within 2 to 6 hours after exposure to virulent B strain, the pigs had cleared the challenge organisms (Table 8). One-half hour postinoculation of virulent B strain, cultures of centrifuged nasal wash indicated that the organism was associated with nasal cavity cells in 1 resistant pig and in 2 control pigs. At 2 and 6 hours postinoculation, the organism was associated with nasal cavity cells in the control pigs. The technique apparently lacked sensitivity because more organisms were occasionally present in the supernatant than in the whole nasal washings. This could be explained by the multiplication of the organism during the time which expired between samples taken for the counts of colony forming units from the whole washings and the supernatant. It was not determined if the B. bronchiseptica organism was associated with epithelial, PMN, or mononuclear cells.

Clearance of the virulent IVDL strain 374 did not occur when it was inoculated intranasally into pigs currently infected with the low-virulence D-1 strain (Experiment IX).

Apparently, the D-1 strain must be completely eliminated from the nasal cavity before the resistance mechanism becomes effective.

Attempts to modify an isolate of D-1 strain by 21 passages on artificial media were disappointing (Experiment X). Although the atypical colony form of D-1 strain was cleared in approximately 5 weeks, resistance to reinfection by virulent B strain was not induced. The 21st passage of D-1 strain did not clear as fast as the low-passage D-1 strain from the nasal cavity of pigs and the factors associated with its ability to produce resistance were lost.

Three types of preparations were injected parenterally in attempts to produce resistance to B. bronchiseptica nasal infection: these were sonicate-vaccine, pertussis-vaccine and whole cell-vaccine. Ganaway et al. (1965) reported that in guinea pigs both pneumonia and infection of the lungs by B. bronchiseptica could be prevented by intramusclar injection of formalized bacterin emulsified in Freund incomplete adjuvant. Winsser (1960) reported that in mice both mortality and infection of the lungs by B. bronchiseptica could be prevented by the intraperitoneal injection of whole cells inactivated with merthiolate. By contrast, intramuscular injection of swine with a whole cell-vaccine (B strain) without adjuvant did not induce nasal resistance against virulent B strain challenge (Experiment XVI).

Because of the failure of the whole cell-vaccine (B strain) to induce resistance, it was postulated that exposure of the pig to the internal antigens of B. bronchiseptica may be necessary for the production of resistance to nasal infection. In preliminary experiments (XI and XII), the sonicate-vaccine (prepared from D-1 strain) was found to protect against intranasal challenge by virulent B strain. However, it was apparent that the type of resistance was different from that produced in the live low-virulence D-1 strain cleared swine. The vaccinated pigs became actively infected with the virulent B strain organisms and did not begin to clear the infection for 21 to 35 days after intranasal inoculation. These experiments also indicated that approximately 30 days must elapse after the last subcutaneous injection of vaccine before resistance to virulent B strain infection would result.

Experiments XIII and XIV were conducted to determine the optimum age for vaccination and the duration of resistance induced by the sonicate-vaccine and the pertussis-vaccine. The ability to induce nasal resistance to B. bronchiseptica in swine with either the sonicate- or pertussis-vaccines was not dependent on the age of the pig at the time of initial injection since resistance was successfully produced in pigs from 2-8 weeks of age (Table 11).

Pigs, which were inoculated intranasally with the virulent organism 35 to 42 days after the first injection of the

vaccines, were resistant in 5 of 12 cases in sonicate-vaccine injected and in 4 of 12 cases in pertussis-vaccine injected pigs when nasal secretions were cultured 35 to 40 days after the first intranasal inoculation of virulent B strain (Table 11). The cultures of nasal secretions collected 61 to 69 days after the first intranasal challenge indicated that resistance was produced in 9 of 10 cases in sonicate-vaccine injected and in 9 of 12 cases in pertussis-vaccine injected pigs (Table 11). A high percentage of the unvaccinated infected control pigs nasal secretions were consistently positive for the organism.

In pigs which were inoculated intranasally with the virulent organism 116 to 120 days after the first injection of the vaccines, resistance was produced in 2 of 4 cases in sonicate-vaccine injected and in 3 of 4 cases in pertussis-vaccine injected pigs when nasal secretions were cultured 17 days after the first intranasal inoculation of virulent B strain (Table 12). The results of cultural examination of nasal secretions collected 47 days after the first intranasal challenge indicated that resistance was produced in all of the sonicate- and pertussis-vaccine injected pigs (Table 12). These results indicate that increasing the time between injection of the vaccines and intranasal challenge with the virulent B strain elicits a more rapid clearance of the organism from the nasal cavity, and that a higher percent of the vaccinated animals are protected against infection as this time interval increases.

Statistical analysis revealed that both the sonicate- and the pertussis-vaccine induced significant levels of resistance to intranasal challenge with virulent B. bronchiseptica (Table 13).

Protection of swine against infection by B. bronchiseptica by injection of a vaccine prepared from B. pertussis is consistent with previous reports concerning cross immunization studies in other animals. Evans and Maitland (1939) injected guinea pigs with heat killed suspensions of B. pertussis and intranasally inoculated them with B. bronchiseptica. Prevention of mortality and lung infection caused by the organism was demonstrated. Eldering (1942) injected mice subcutaneously with merthiolate inactivated whole cells of B. pertussis and then challenged the mice by intraperitoneal inoculation of B. bronchiseptica. Prevention of mortality was demonstrated (Table 1). By contrast, Winsser (1960) attempted immunization of mice with merthiolate inactivated B. pertussis whole cell vaccines injected intraperitoneally. The vaccinated mice were not protected against mortality caused by the intranasal inoculation of B. bronchiseptica, and the organism was recovered from the lungs. Kendrick et al. (1953) and Ross et al. (1969) reported that the intraperitoneal injection of B. pertussis whole cell vaccines protected mice against mortality caused by the intracerebral inoculation of B. bronchiseptica.

The protection of swine against infection by B. bronchiseptica by the injection of a vaccine prepared by the sonication

of the organism was comparable to the immunization studies in mice conducted by other workers. Eldering (1941) injected mice subcutaneously with lipopolysaccharide fractions of B. bronchiseptica and challenged with the organism by intraperitoneal inoculation. Prevention of mortality was demonstrated (Table 1). Ross et al. (1969) injected mice intraperitoneally with a saline extract of mechanically disrupted B. bronchiseptica and demonstrated protection against mortality when the mice were challenged intracerebrally with the organism.

The protective antigens contained in the lipopolysaccharide fractions of Eldering (1941) and in the saline extract of Ross et al. (1969) were possibly the same as the antigens present in the sonicate-vaccine. The results of the current study of the active immunization of pigs by the injection of vaccines indicate that vaccines similar to those with the capability of protecting mice against mortality somehow induce a resistance to infection of the swine nasal cavity by B. bronchiseptica. This resistance to infection, however, was not manifested by a complete refractiveness to the challenge organisms soon after intranasal inoculation since 40 to 60 days were required for the clearance of virulent B. bronchiseptica. This was not the case in the low-virulence D-1 strain cleared resistant swine since they were capable of elimination of virulent B. bronchiseptica in 2-6 hours after challenge. Apparently, the vaccine does not prevent infection but actually allows the pig to

develop a resistance which eliminates the infection faster than in unvaccinated control pigs. Possibly, the correlation between protection against mortality in mice and nasal resistance to infection in swine can be explained by postulating that the vaccine actually prevents damage to the respiratory mucosa which allows the pig to eliminate the infection by activation of its own local respiratory tract defense mechanisms.

Dolby et al. (1961) demonstrated that B. pertussis was present initially but disappeared from the lungs of immunized mice within 28 days after vaccination. These mice were immunized with a vaccine which protected against mortality caused by intracerebral inoculation. Such results are consistent with the current observations on nasal resistance in swine immunized with either sonicate- or pertussis-vaccine.

The attempts to clear virulent B strain infection from swine by injection of pertussis-vaccine 7 days after initial intranasal exposure were not successful (Experiment XV). As in the case of the D-1 strain cleared resistance, it appears that the pig must be free of the virulent strain at the time of vaccination for successful development of resistance.

As discussed earlier, the trial (Experiment XVI) using a formalin inactivated whole cell-vaccine prepared from B strain to protect pigs against intranasal inoculation of B strain did not produce resistance. This finding is inconsistent with the results of Ganaway et al. (1965) and Winsser (1960). Therefore,

Experiment XVII was planned to supplement the observations in the previous experiment. In Experiment XVII, the 4 swine injected subcutaneously with a formalin inactivated whole cell-vaccine prepared from D-1 strain cleared the virulent B strain by 60 days after the first exposure to the challenge organisms (Table 14).

Several differences existed between Experiment XVI and XVII any one of which could explain the variation in results. The whole cell-vaccines were prepared from 2 different strains with the same concentration of formalin used for inactivation. However, the B strain bacterin was inactivated by incubation at room temperature and the D-1 strain bacterin was inactivated by incubation at refrigerator temperature. The B strain bacterin was not mixed with adjuvant and was injected intramuscularly. The D-1 strain bacterin was mixed with Freund incomplete adjuvant and was injected subcutaneously. The interval of time elapsing between the last injection of the B strain bacterin and challenge was 8 days while 32 days elapsed in the case of the D-1 strain bacterin. Additional trials will be necessary to clarify the variation in the results of these 2 experiments.

Bordetella bronchiseptica may infect the pig nasal cavity as early as 48 hours after birth (Harris and Switzer, 1968). The types of resistance which have been described thus far require a time interval of several days before challenge by

virulent organisms and do not clear the organism if immunization is attempted during an active infection. Since these types have serious practical limitations, an attempt was made to produce passive resistance by immunization of pregnant sows.

Results of Experiments XVIII, XIX, XX, and XXI indicated that sonicate-vaccine which was capable of eliciting active nasal resistance in young pigs would not induce either resistance to intranasal infection by virulent B strain or prevent the development of lesions in the respiratory tract of passively immunized pigs (Tables 15 and 16). There was, however, a decrease in the number of tracheas and lungs positive for the organism in pigs from vaccinated sows as opposed to unvaccinated sows. Five of 12 pigs from unvaccinated sows had lesions of pneumonia while 0 of 14 pigs from vaccinated sows had lung lesions. The mechanism which allows production of nasal resistance in pigs following active immunization was not induced in passively immunized pigs born of sows injected with sonicate-vaccine.

The trial comparing the influence of the age of pigs at the time of first intranasal exposure to virulent B. bronchiseptica B strain on the duration of infection is of interest (Table 17). The 24-week-old pigs became infected with the organism but cleared the infection by 33 days postinoculation at which time the 3-week-old pigs were still infected. Because the clearance of the B strain from 24-week-old pigs resembled

the clearance of D-1 strain from young pigs, 2 of the B strain cleared pigs were challenged with B strain organisms. The nasal secretions of these pigs were negative for the organism 7 days postinoculation; however, the secretions contained the organism when the pigs were necropsied 9 days postinoculation. This suggests that clearance of B strain from the nasal cavity may not result in the inducement of nasal resistance of the magnitude elicited by the clearance of D-1 strain.

The results of Experiment VI indicated that some mild damage to the turbinate was caused by the low-virulence D-1 strain infection; however, the damage was mild as compared to infection by virulent B strain. The cellular response occurring in the lamina propria of the turbinates of D-1 strain infected and/or cleared swine consisted of a mild diffuse infiltration of mononuclear cells. When these pigs were cleared of the D-1 strain infection and then inoculated intranasally with virulent B strain, focal accumulations of mononuclear cells were present in the lamina propria of resistant pigs when necropsied 1 week postinoculation (Experiment V). The epithelium of these resistant pigs appeared normal. The turbinates of the control swine which were inoculated intranasally with B strain and necropsied at the same time did not contain mononuclear cell infiltrate but had hyperplasia of the epithelium.

In other experiments, the positive control swine were not necropsied until 30 to 60 days after inoculation of the virulent B strain. In many of these pigs, focal accumulations of mononuclear cells occurred in the lamina propria which was consistent with the observations of Duncan (1965). In the experiments in which resistance was produced by injections of either sonicate- or pertussis-vaccines, massive focal accumulations of mononuclear cells occurred in the turbinate lamina propria of most pigs. In some pigs these accumulations resembled lymph node follicles. A majority of mononuclear cells in the focal accumulations were pyroninophilic in either positive control or resistant pigs. Occasionally in both positive control and resistant pigs the mononuclear cells were observed in close association with the epithelium and appeared to be migrating through the epithelium. The pyroninophilic nature of these cells would indicate active protein synthesis associated either with cellular proliferation or with the production of proteins for humoral secretion. Berenbaum et al. (1960) reported that the brains of pertussis vaccinated mice contained an intense focal mononuclear cell response which was associated with the elimination of B. pertussis infection after intracerebral challenge inoculation.

Possibly, focal accumulation of monocytes is associated with an immunologic mechanism which may explain the rapid and complete resistance in the D-1 strain cleared swine, the

development of the ability to clear the infection in sonicate-vaccine and pertussis-vaccine injected swine, and the gradual clearance of the infection in positive control swine. This immunologic mechanism could be of a cellular and/or humoral nature.

The antibody titers present in the serum of pigs during the course of an experiment were determined by the PAST. Titers of antibody were detectable from the serum of pigs which were infected with low-virulence D-1 strain B. bronchiseptica but were rarely higher than 32 and some pigs did not have a detectable level. The serum titers of antibody in resistant pigs were not increased upon challenge inoculation with virulent B strain. B strain infected pig serum antibody titers were of the same magnitude as the antibody titers from pigs which were infected with the D-1 strain.

The serum antibody titers of the positive control pigs in Experiment XIII indicated that higher responses were stimulated by increasing the age of the pigs at the time of the first intranasal inoculation with the virulent B strain (Figure 4). The titers tended to peak at 2 week postinoculation and to decrease from then on. The sera of the baby pigs in Experiments XVIII and XIX exhibited titers up to 32 at 3 days of age before intranasal inoculation of the virulent B strain which was attributed to a non-specific reaction since sera from these pigs then consistently remained negative for antibody for several weeks postinoculation.

The detection of antibody in the sera of experimentally infected pigs was consistent with the observations of Ross (1965), who, (as in Experiments XVIII and XIX when 3-day-old pigs were inoculated with virulent organisms) did not detect agglutinins in the serum of pigs until 20 weeks postinoculation while pigs inoculated with virulent strain at 4 weeks of age, developed detectable agglutinins with the tube agglutination test at 6 to 8 weeks postinoculation.

High serum antibody titers were present in pigs injected with sonicate- and pertussis-vaccine, with whole cell-vaccines (B and D-1 strain) and in pigs receiving colostrum from sows injected with sonicate-vaccine (Figures 4, 5, 8, and 9). The serum antibody titers in sonicate- and pertussis-vaccine and in D-1 strain whole cell-vaccine injected pigs remained rather constant during infection with virulent B strain. At the time of clearance of the infection the titers were usually increased. However, this was not the case with pigs injected with sonicate-vaccine at 2 weeks of age (Figure 4).

The results of the determinations of the antibody titers with the PAST indicate that serum antibody was not associated with resistance to intranasal infection. This was evident because low or undetectable levels of antibody were present in the D-1 strain cleared swine which were resistant to challenge inoculation. By contrast, the pigs receiving sonicate- and

pertussis-vaccine had high serum antibody titers but became infected initially when inoculated intranasally with virulent B strain. It seems likely that the agglutinogen(s) are not responsible for induction of protection. By comparison, Holt and Spasojevic (1968) reported that vaccines, prepared from B. pertussis strains which did not possess agglutinogens, protected mice against intracerebral challenge by B. pertussis.

Antibodies were demonstrated by the PAST in concentrated nasal washings collected from pigs cleared of D-1 strain, from D-1 cleared pigs which had resisted B strain challenge, from pigs infected with B strain, and from pigs injected with sonicate-vaccine both before and after B strain challenge (Table 25). With the detection of antibody in the nasal washings, an effort was made to determine the significance of these antibodies and to associate them with resistance to infection by B. bronchiseptica.

It was demonstrated in vitro that swine nasal epithelial cells attached to the B. bronchiseptica organism with the formation of rosettes of erythrocytes around the epithelial cells. It appeared that the organisms were bridging and connecting the epithelial cells to the erythrocytes. It is believed that Tartakowsky (1898) first described the close affinity with which the B. bronchiseptica cells adhered to the respiratory tract epithelium of the guinea pig (Smith, 1913). However, no in vitro studies with either B. bronchiseptica or

B. pertussis have been reported which have assessed the role of specific attachment as an invasive mechanism.

The attachment to tracheal epithelial cells by Mycoplasma pneumoniae has recently been demonstrated (Sobeslavsky et al., 1968 and Lipman and Clyde, 1969). Sobeslavsky et al. (1968) reported that the receptors on erythrocytes were neuraminic acid and that the same receptor sites for M. pneumoniae were present on the tracheal epithelial cell. Lamb and Reid (1968) demonstrated sialomucin in rat respiratory tract secretions. Antibody or sialomucin may have the capacity to block the attachment sites present on certain infectious agents and prevent their attachment to respiratory tract epithelial cells. The attachment site blocked infectious particles could then be eliminated as inert particles by the mucociliary apparatus.

Based on this information, it was postulated that substances may be present in the nasal washings from pigs which would inhibit agglutination of erythrocytes by B. bronchiseptica. Such an inhibition of attachment to nasal and tracheal epithelial cells would be a mechanism which would explain the production of resistance to infection.

The direct hemagglutination-inhibition test was performed with the concentrated nasal wash samples. Substances were present which inhibited agglutination of erythrocytes in the nasal washings of D-1 strain cleared pigs, of D-1 cleared and exposed to B strain pigs, of B strain exposed pigs, and of pigs

injected with sonicate-vaccine and exposed to B strain (Table 25). The direct hemagglutination-inhibition test was quite variable on repeat determinations. Some nasal washings from unexposed, unvaccinated swine contained hemagglutination-inhibiting substances; however, the titers of the inhibitory substances from infected or resistant swine were of a higher magnitude.

The data indicating the presence of antibody either by the PAST or the HAI test in nasal washings was considered to be too preliminary for final conclusions. The possibility that substances may be stimulated in resistant or infected pigs which inhibit the attachment of B. bronchiseptica to the epithelial cells needs further investigation. For example, it would be logical for the B strain infection of pigs to stimulate substances (antibody) which would inhibit epithelial cell attachment. However, if the mucociliary apparatus was damaged due to an infection, then the substances would not prevent colonization of damaged, cilia denuded, portions of the mucosa. In the D-1 cleared pigs, when the mucociliary apparatus was quite functional, substances could have inhibited epithelial cell attachment by the organism. The organism would then be removed by the host's natural defense mechanism, the mucociliary apparatus.

A similar explanation for the resistance induced by sonicate- and pertussis-vaccine and the D-1 strain whole cell-vaccine can be proposed. In such types of nasal resistance,

the action of the vaccines would be to prevent damage caused by the organism to the mucociliary apparatus. In this way, the presence of the organism for 30 to 60 days following intranasal inoculation would stimulate the inhibitory substances necessary for prevention of attachment to the epithelial cells. The above working hypothesis is suggested to aid future research on the definition of the mechanism of nasal resistance to B. bronchiseptica infection.

This hypothesis gains indirect support from experiments conducted in mice with regard to resistance to lung infection by B. pertussis. Dolby and Dolby (1969) injected rabbits with B. pertussis vaccine. Fractions of the antisera were incubated with inoculum which consisted of lethal numbers of organisms for intracerebral challenge and of sub-lethal numbers of organisms for intranasal challenge in mice. Resistance was evaluated by brain and lung counts of B. pertussis. The serum fraction associated with the maximum bactericidal activity in vitro conferred resistance to intracerebral challenge. However, the fraction associated with hemagglutination-inhibition activity in vitro was correlated with resistance to intranasal challenge.

Masry (1952) reported that the passive injection of anti-hemagglutinin would not protect mice against mortality when challenged with lethal doses of B. pertussis either by the intracerebral or intranasal route. However, this method of

administration would not necessarily result in anti-hemagglutinin being present in the respiratory secretions. Also, such receptor blockage by anti-hemagglutinin would not be of value in the intracerebral challenge test.

These results of Masry (1952) have received considerable prominence as indicators that anti-hemagglutinin is not involved in protection of mice against intranasal challenge. The results obtained in the present study suggest that this classical work must be reevaluated. Anti-hemagglutinin, when present in nasal secretions, may be a major resistance mechanism to protect the nasal epithelium against Bordetella attachment and colonization.

Experiments were conducted with pooled serum and nasal washings using the opsonocytophagic test and the bactericidal test (Tables 26 and 27). Serum and nasal wash samples containing antibody to B. bronchiseptica, detectable by the PAST with D-1 strain as the test organism, gave higher phagocytic index and percent phagocytosis values than with B strain as the test organism. The D-1 strain appeared to be a more sensitive indicator than B strain for detecting the presence of opsonins from various samples.

The pooled serum samples from pigs injected with the 4 types of vaccines increased the phagocytic index (D-1 strain) more than 12 units as compared to the serum from unexposed, unvaccinated pigs. The pooled serum samples from D-1 strain

cleared pigs, and from D-1 strain cleared and exposed to B strain pigs increased the phagocytic index (D-1 strain) by more than 6 units as compared to serum from unexposed, unvaccinated pigs. By contrast, serum from pigs injected with B strain whole cell-vaccine and sonicate-vaccine increased the phagocytic index (B strain) by only 6 units. All other sera tested produced less than 6 units difference as compared to unexposed, unvaccinated pig serum for the phagocytic index conducted with B strain as the test organism.

One of 3 pooled nasal samples from D-1 cleared and exposed to B strain pigs, and 1 of 1 comparable samples from pigs injected with sonicate-vaccine increased the phagocytic index (D-1 strain) by more than 3 units as compared to nasal wash samples from unexposed, unvaccinated control swine. Very small increases in the phagocytic index occurred when B strain was used as the test organism.

It was concluded that opsonins present in the sera or nasal washings of the D-1 strain cleared and vaccinated swine were not correlated with respiratory resistance. This was illustrated by the rather high titer of opsonin in the sera from pigs injected with B strain whole cell-vaccine even though the pigs were not resistant to challenge inoculation. Also, the pooled nasal wash from sonicate-vaccine injected pigs contained a high titer of opsonin before challenge with virulent B strain. Such pigs become infected initially when challenged with B strain.

The pooled serum sample from pigs injected with B strain whole cell-vaccine was bactericidal as compared to the serum from exposed, unvaccinated pigs (Table 27). The pooled nasal wash sample from D-1 strain cleared and B strain exposed pigs was not bactericidal when used either in the presence or absence of swine complement. Based on these preliminary experiments, it appeared that bactericidal substances were probably not involved in the induction of resistance to nasal infection by B. bronchiseptica. However, more trials with a greater number of samples would be necessary before reaching a definite conclusion.

Direct hemagglutination by various respiratory tract pathogenic bacteria was investigated because these organisms may attach to the respiratory mucosa by the same mechanism involved in erythrocyte agglutination. Bordetella bronchiseptica and Haemophilus spp. readily infect the swine nasal cavity when inoculated into experimental swine. Pasteurella multocida does not readily establish a primary infection. Harris and Switzer (1968) have shown that preconditioning of the nasal epithelium with B. bronchiseptica infection results in the establishment of P. multocida infection. The results of the hemagglutination studies indicate a correlation between the ability of a microorganism to establish a primary infection and to agglutinate erythrocytes. Further research characterizing the nature of the attachment sites on epithelial cells

should be conducted to expand and develop this preliminary observation since it may be of prime importance in understanding the pathogenesis of bacterial infections of the respiratory tract.

It is of interest that the low-virulence D-1 strain possessed more hemagglutinin per cell than the virulent B strain. This could allow an increased local production of anti-hemagglutinin in the nasal secretions of the resistant pigs which have cleared the D-1 strain. The present studies have also demonstrated that B. bronchiseptica agglutinates swine, human, sheep, and guinea pig erythrocytes and that B. pertussis agglutinates swine and human erythrocytes. It has been previously reported that B. bronchiseptica and B. pertussis agglutinate human, mouse, and fowl erythrocytes (Keogh et al., 1947). It has also been reported that B. bronchiseptica agglutinates sheep (Joubert et al., 1960), horse and parakeet (Gallagher, 1965) erythrocytes.

The results of the phagocytosis studies of the 2 strains of B. bronchiseptica by swine PMN leucocytes indicated a statistically significant increase (at the 0.01 probability level) in the phagocytic index obtained with the low-virulence D-1 strain as opposed to the virulent B strain (Table 28). The PMN leucocytes present in the nasal cavity of the pigs infected with the D-1 strain should more effectively remove this organism than the B strain from the mucosa. This may be the

mechanism responsible for the natural clearance of this strain from the nasal cavity more rapidly than the B strain.

An attempt was made to critically evaluate the Particulate Antigen Settling Test (PAST) for its usefulness as a routine serological test. Initially, a problem with non-specific positive reactions occurred with sera from unexposed, unvaccinated pigs. The non-specific reaction could be decreased by the use of 5 percent NaCl as diluent without appreciable reduction in the antibody titers of serum from either infected or vaccinated pigs (Table 18). Ninety-three serum samples from unexposed, unvaccinated swine of various ages were titrated for antibody during this study (Table 19). Eighty of these samples contained non-specific reactions at the 1 to 4 dilution or less. Pigs which were 3 days of age, or over 16 weeks of age had sera with the most non-specific reactions. Antigens prepared in broth, either inactivated or live, appeared to be the most sensitive indicator of antibody titer (Table 20). The formalin inactivated antigen prepared from D-1 strain was utilized because of its greater stability as compared to live antigen preparations and its increased sensitivity over antigens prepared from B strain.

The reproducibility within lots and between different lots of D-1 strain inactivated broth antigen was determined (Table 21). The reproducibility within a lot of antigen usually varied by less than a 1-fold dilution. The reproducibility

between lots of antigens was more erratic in that a 4-fold dilution variation occurred with some serum samples.

The comparison of the titers of swine and rabbit sera in the tube, plate, PAST, and MAT revealed that the PAST was more sensitive than the other types of test (Tables 22 and 23). Swine or rabbits which had been intranasally exposed to or received injections of B strain B. bronchiseptica exhibited the greatest difference in titers, ranging from 3- to 6-fold dilutions. The difference in titers in animals receiving homologous antigen exposure (D-1 strain) were increased by 2- to 6-fold dilutions. These results disagree somewhat with those reported by other workers. Beneson et al. (1968) reported that antibody titers in human patients infected with Vibrio cholerae were lower by 2- to 3-fold dilutions in a similar microtechnique test than in the tube agglutination test. Their results indicated that sera tested with a heterologous antigen gave a similar titer in either test.

The comparison of titers present in swine and rabbit sera containing antibody to Salmonella spp. antigens by the tube, PAST, and MAT indicated a very small variation in titer between tests (Table 24). It was concluded that the reason for the increased sensitivity of the PAST with B. bronchiseptica antigens was because the agglutinated clumps of antigen were mechanically broken up in the reading of the tube test and the MAT, whereas in the PAST, the antigen and antibody settled to

the bottom of the well and was not disturbed physically before reading the test.

Another reason for the increased sensitivity was explained by the comparison in which the serum was diluted with either pipettes or microdiluters (Table 22). Some serum samples had a 4-fold increase in titer by the use of the microdiluters as compared to pipette dilutions in the PAST. Hirata et al. (1969) has reported that microdiluters may cause exaggerated passive hemagglutination titers.

The PAST was used for the detection of infection by B. bronchiseptica in experimental swine (Table 23). Of the 21 unvaccinated, exposed control swine serum samples, 20 pigs contained titers of 8 or more in the PAST as compared to only 2 samples with titers detectable in the plate agglutination test. Utilization of the PAST for detection of infected pigs in the field is a possibility. However, the detection of the nasal carriers of B. bronchiseptica is more easily accomplished by bacteriologic culturing.

ACKNOWLEDGEMENTS

This dissertation could not have been accomplished without guidance, counsel, encouragement, and assistance. I express sincere gratitude to Dr. W. P. Switzer for his fulfillment of all four of the above criteria. I am deeply appreciative to my brother, Dr. R. A. Harris, for his constant antagonism during our childhood and for his constant encouragement during our professional careers.

I thank Drs. R. F. Ross and J. R. Duncan for their research on Bordetella rhinitis upon which much of this dissertation was based.

I am appreciative for the administrative foresight and ability of Dr. E. E. Wedman which enabled me to obtain monetary support for my graduate program. I thank the faculty of the Veterinary Medical Research Institute for their interest and assistance.

I sincerely express appreciation to my wife, Judirae, who has worked full-time during my undergraduate, professional, and graduate educational programs. I thank my children, Wendi and Susan, for their understanding of my family absenteeism during this time.

I am grateful to my friend, Dr. R. D. Glock, for his social companionship during my attacks of acute depression.

The technical and animal caretaking assistance of the following people are greatly appreciated: Mrs. I. H. Hegland, Mrs. R. I. Burroughs, Mrs. C. A. Jotzke, Miss L. VanSanten, Mrs. J. M. Kinyon, Mrs. E. Campbell, Mr. J. Oppedal, and my father, Mr. A. E. Harris.

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